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PROPERITES OF AN R FACTOR SPECIFIC BACTERIOPHAGE

by

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TO THE MEMORY OF MY FATHER

ABSTRACT

A new bacteriophage PR5 was isolated and some of its biochemical and biological properties were studied.

The phage appears to have an icosahedral structure which sometimes bears a tail. The phage particles have a buoyant density of 1.26 g/ml in CsCl. Its nucleic acid content is composed of double-stranded linear DNA molecules which have a molecular weight of 7.4×10^6 daltons and a G+C ratio of 49%. The phage has 81 parts of protein to 19 parts of DNA by weight. Approximately 17.5% of the dry weight of phage material was lipid. The chief lipids present are phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin.

The phage is specific to the P1, W and N groups of plasmids and has a wide bacterial host range. Different burst sizes are observed with different host bacteria. Adsorption of phage to bacteria appears to be somatic. Studies using bacterial hosts defective in lipopolysaccharide structure suggests that the receptor involves hexose sugars of the core lipopolysaccharide.

Results of serological studies have established PR5 as a different phage form two other related phages PR3 and PR4.

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INTRODUCTION

In 1960, Loeb reported the isolation of a series of male specific phages which he labelled f1 through f7 because of their specificity for Escherichia coli strains carrying the fertility factor F (47). Since then, other male phages have been isolated in various laboratories through out the world. They constitute a unique class of bacterial virus which share one host range peculiarity- these phage can only infect those bacteria carrying a sex factor, and do not grow on female (F−) bacteria. However, except for this common host specificity, they represent a rather heterogeneous group with respect to size and shape, nucleic acid content and other properties. In view of these diversities, they can be classified into 3 groups: (1) isometric RNA phage, (2) filamentous DNA phage, (3) isometric DNA phage. A possible fourth group which corresponds to filamentous RNA phage has not been described.

1. Isometric RNA phage

An especially large number of independent isolates come under this group. The most studied are coliphages like f2, R17, MS2, and QB . Transfer of the F factor to strains of Shigella , Salmonella , and Proteus brings these bacteria within the host range of the phage. New isolates are continuously being described. These include M12 (34); fr (54); β (65); Fh5 (28); u2 (16); f can (21); some 30 phages reported in Japan (94); 6 isolates by Bishop and Bradley

(6); and a recent isolate PRR1 by Olsen (67).

These isometric RNA phage contain single-stranded RNA molecules. They appear to be tailess and have a more or less similar hexagonal outline by electron microscope which suggests an icosahedral structure. Different authors have reported variation in the number of capsomeres with different phage (10,92).

Watanabe studied the serological relationship of these phages and found that they fall into 3 groups (94). He further demonstrated that serologically related phages also have similar RNA base ratios (95), membrane filter-salt elution patterns (63), and buoyant densities in CsCl (64). Studies in other laboratories also indicated relatedness among these phages with the exception of QB (69,80). The 6 isolates of Bishop and Bradley were found to fall into 2 serological groups (6). Base ratio analysis showed that two members of one group, ZJ/1 and ZIK/1, were similar to QB, while the remaining group was similar to the f2 phages (70). Serological relationships of the R factor phage PRR1 with these RNA phages have not been investigated. However, it was shown to have a different salt elution pattern on Millipore filter from f2, which suggests dissimilar coat protein composition (67).

Attachment of RNA phages to their host occur along the side of sex pili (15,37). Further more, with the exception of PRR1, they all adsorb to F pili or F-like pili produced by certain R factors which are antigenically different from

the I or I-like pili produced by some colicinogenic factors and R factors (60). In fact these phages are often used to determine the nature of sex pili generated by various sex factors. The specific nature of the pili which adsorb PRR1 has not been reported.

Intracellular particles can be seen in infected host bacteria. Phage assembly appeared to be cytoplasmic (10). These RNA phages generally kill their hosts after infection and phage progeny is released by cell lysis.

Table I lists some properties of the RNA phages which have been studies. They are grouped after Watanabe's scheme which is based primarily on their serological relatedness, similarity in base ratios, and density in CsCl. Phages which were not included in the original studies have been added to each group based on published date.

2. Filamentous DNA phage

Shortly after the discovery of the RNA phages, 2 reports appeared describing the isolations of two rod-like DNA-containing male phages fd and M13 (54,34,). Meanwhile one of the seven phages isolated by Loeb was also found to be filamentous. Other phage isolated under this category are shown in table II along with some of their properties.

These filamentous phages are long flexible filaments of 5.5 nm in diameter. Two length distributions have been observed, the F phages being 850 nm long and the Pseudomonas

TABLE I

RNA PHAGES

Phage	Diameter (nm)	Buoyant Density	S20,W	%RNA	Mol Wt $x10^6$	Base Ratio C	A	U	G
MS2	26 (87)	1.46 (87)	81 (87)	32 (87)	1.05 (87)	25.3 24.5	23.2 22.4	24.8 25.6 (87)	26.7[1] 27.5[2]
f2	20 (78)					25.9	22.1	25.1 (48)	26.8
R17	26.6 (27)		78.9 (24)	31.7 (24)	1.1 (30)	24.9	23.1	25.7 (62)	26.3
β			75–90 (65)			27.2	23.2	28.0 (65)	21.6
fr	21 (54)	1.46 (54)	79 (54)	28.4 (55)	1.2 (55)	24.9	24.3	23.7 (55)	27.1
ZG[3]	22.5 (6)					25.1	24.0	23.7 (6)	27.2
M12	27 (34)				1 (84)				
u2	24 (16)	1.42 (16)	78 (16)	32-34 (16)		23.7	23.8	27.1 (16)	25.4
MY[4]		1.457 (95)	79 (95)			25.9	23.3	24.6 (95)	26.2[5]
PRR1	25 (67)	1.453 (67)	80 (67)						
GA[6]		1.44 (95)	76 (95)			23.9	27.8	23.7 (95)	24.6[7]
QB[8]	25 (69)	1.47 (69)	83 (69)			22.1	29.1	23.7 (69)	24.7
ZJ/1[9]	22.5 (6)					23.7	28.2	23.8 (6)	24.3

[1] Acid hydrolysis

[2] Alkaline hydrolysis

[3] ZG is similar to 3 other isolates by Bishop and Bradley

[4] MY is similar to 7 other isolates in Japan

[5] Figures calculated from reference (95)

[6] GA is similar to 8 other isolates in Japan

[7] Figures calculated from reference (95)

[8] QB is similar to 11 other isolates in Japan

[9] AJ/1 is similar to ZIK/1 isolated by Bishop and Bradley

TABLE II

FILAMENTOUS DNA PHAGES

Phage	Pili Specificity	Length nm	Diameter nm	Buoyant Density	S20,W	%DNA	Mol Wt x10^6	Base Ratio			
								T	C	A	G
fd	F	760 (54)	50 (54)	1.36 (33)	40 (54)	12.2 (33)	1.37 (33)	34.1	21.7	24.4 (33)	19.9
f1	F	850 (98)	43-63 (98)	1.3 (98)		11.1 (98)					
M13	F	800 (75)	60 (75)	1.29 (75)				35.8	19.8	23.3 (75)	21.1
If1	I	1300 (58)	55 (58)		45 (58)	12 (58)	3 (58)				
Pf1						12 (89)					
ZJ/2	F	830 (8)	68 (8)								
Ec9	F	600 (14)	50 (14)		39 (14)	14.8 (22)		34.4	20.7	24.4 (14)	20.6
AE2	F	800 (35)	50 (35)		43 (35)		14 (35)	32	21	26 (35)	21
A	F	830 (58)	60 (58)	1.30 (58)	46 (58)		10 (58)	33.1	20.0	25.7 (58)	21.2
HR	F	800 (35)	60 (35)	1.31 (35)							

and I phages being 1300 nm. X-ray diffraction patterns showed that the coat protein molecule is arranged in the form of an α helix (56). The nucleic acid is a single-strand of circular DNA formed into a linear molecule like a circle of string pulled taut at opposite ends (57).

Most filamentous phages adsorb to the tips of sex pili. Thus, formation of mating pairs and phage adsorption interfere with each other (37,68). Since there are only a few of these pili per bacterium, the adsorption rate of these phages is low. Experiments using sex pili sheared off with a blender showed that adsorption is polar in nature, that is, only one end of the phage filament is used to adsorb to a pilus, and only one end of the pilus can adsorb the phage (58). Unlike the RNA phage which adsorb exclusively to the F type of pili, these filamentous DNA phages exhibit different pili specificities. The majority adsorb to F type of pili while the If phages can only adsorb to I type pili. The phage Ike, however, does not adsorb to either of these pili types. No pili receptor site was detected with this phage (40). The same is true with the _Pseudomonas_ phage Pf. No pilus + phage complexes have been demonstrated from sensitive hosts although these hosts apparently formed recombinants with female bacteria (11,61).

Unlike the RNA phages which eventually lyse their hosts, these filamentous phages are released from infected bacteria without killing them. There is a pool of viral DNA in the cell, but no viral protein has been detected in the

cytoplasm. It has been suggested that the viral DNA picks up its coat protein from the cell membrane while being extruded (9,79). However, single gene mutation in the phage genome did give rise to mutants which cause bacterial lysis. They stop the growth of the host and destroy its colony forming ability (73). Filamentous DNA phages are notorious for their turbid, ill-defined plagues due to bacterial growth.

Serum neutralization studies established the If phage and Pf phage as 2 distinct groups which are in turn different from the remaining group of F phage (35,59,61,73). Antisera raised specifically to one of the If, Pf or fd group of phage apparently have little or no inactivating activity for members of the other two groups. However, interpretation of these results is complicated by electron microscopic studies which revealed that antibodies to If did adsorb to the side of fd and Ec9 filaments, although apparently this had no effect on the plague forming ability of these viruses (59). On the other hand, studies with M13 mutants showed that changes in the coat protein (B protein) of the phage altered its sensitivity to antiserum (58). Thus, it is clear that antigenic behavior does not offer a straight-forward answer as to the relatedness of these phages.

3. Isometric DNA phage

The detection of this group of male phage occurred only

recently. So far only three phages are known to belong to this group, designated PR3, PR4 and PR5. PR5 was isolated during an unsuccessful effort to search for a phage specific for the P2 group of R factors (81). While PR5 was being characterized, another report appeared which also described the isolation of two similar phages PR3 and PR4 in Australia (85). Since preliminary studies revealed some interesting features about these phages and little has been published about them so far, it is the purpose of this thesis to describe some biological and biochemical properties of the member of the group detected locally, phage PR5.

The isometric DNA phages appear in electron microscope as icosahedra some 65 nm in diameter, with or without a tail (12, this thesis). One outstanding feature of the virus is their thick double walled appearance which resembles the lipovirus PM2 (32) and suggests the presence of lipids. Bradley and Rutherford have supported this suggestion by chloroform inactivation of PR3 and PR4 infectivity (12). The nature or quantitation of the lipid material has not been reported. Studies on PR5 reported in this thesis confirm the presence of lipid in PR5.

Phage PR3 and PR4 nucleic acid has been reported to be double-stranded DNA based on fluorescent staining of phage DNA. Further characters of the DNA have not been described.

Unlike the isometric RNA phages whose host range includes bacteria carrying the fertility factor F and certain R factors which generate similar F-like pili, these

DNA phages are strictly R specific, being able to infect only three classes of R factors, and would not infect a bacteria strain carrying a _Pseudomonas_ fertility factor FP (85). The most efficient hosts studied with PR5 are _P. aeruginosa_ strains carrying the R factor RP4.

The R factor RP4 belongs to the P1 group of R factors (81) carrying resistant determinant to tetracycline, neomycin / kanamycin, and carbenicillin. This R factor has been isolated independently and has been variously designated as RP1 (31), RP4 (20), R1822 (66) and R18 (17). Studies on these isolates indicated that they are similar, if not identical R factors. It has an unusually wide host range which extend outside the family _Enterobacteriacaea_ to include _Pseudomonas_ and some soil organisms (20,66). This perhaps explains the somewhat ubiquitous occurence of the PR phages. Unlike many other R factors which either form F-like or I-like pili, RP4 does not produce either, as indicated by the resistance of RP4-carrying bacteria to both F and I phages (20). However, there is evidence that this R factor does produce sex pili (67,12).

So far five phages are known to infect bacteria carrying this R factor. These include the PR5 group of DNA phages, the filamentous DNA phage Pf3, and the isometric RNA phage PRR1. Except for PRR1 which adsorb to pili, the receptor sites of the remaining phages appear to occur on the cell surface over the whole of the bacterium. The only somatic male phages known outside this group are the

Pseudomonas phage Pf and the R factor phage Ike.

Failure to demonstrate sex pili in some male bacteria has remained a puzzle and presents a difficulty to the existing models of bacterial conjugation. The discovery of this group of somatic male phages perhaps make things easier for they indicate that some cell wall antigens other than sex pili are also produced after the bacteria has received the sex factors, and it is not unreasonable to suppose this might also be involved in the process of bacterial conjugation. In addition because the host range of these phages is limited to bacteria which carry FP factors or certain classes of R factors unrelated to the F factors, it is possible that different systems exist depending on the particular plasmids and hosts involved. In any case, these somatic phages might prove to be useful tools for the understanding of bacterial conjugation just as the pili phages have been. The PR5 group of phage will offer particular advantage due to its unusually wide host range.

Another interesting feature of the PR5 group of phage is their lipid content. Relatively few lipid containing phages have been described. Only two of these phages have been studied intensively, one of which is the marine Pseudomonas BAL31 phage PM2 (25) and the other is the Pseudomonas phaseolicola phage ø6 (93). Both are icosahedral in structure, and like PR5, have unusually low buoyant density of less than 1.3 g/cm^3 in CsCl. A list of some properties of PM2 and ø6 are given in table III.

TABLE III

PROPERTIES OF THE LIPID PHAGES ø6 AND PM2

	ø6		PM2	
Diameter of phage particles	60-100nm with envelope 50nm without envelope (23)		61.4nm (83)	
Buoyant density of phage particles	1.29 g/ml (93)		1.28 g/ml (25)	
Phage nucleic acid	Double-stranded RNA (93)		Double-stranded DNA (25)	
% nucleic acid by weight	13 (93)		10-11 (32)	
G+C content of nucleic acid			42-43% (25)	
Mol. wt. of nucleic acid			6 x 10^6 (26)	
% lipid by weight	25 (93)		14-16 (32)	
	Phage	Host	Phage	Host
% phosphatidylglycerol	57	29	65-68	23
% phosphatidylethanolamine	35	56	28	75
% cardiolipin	8	15		
% phosphotidic acid			2-4	
	(76)		(13)	

The two phages have very different appearances by electron microscopy. ø6 was seen to be surrounded by a thick lipid envelope which sometimes give the appearance of a blunt tail, probably a result of streaming of the lipid layer. Removal of this lipid layer with chloroform revealed an icosahedral structure 50 nm in diameter (23). No lipid envelope was observed with PM2. Instead, the viral shell appears double-walled and represents a lipid bilayer (32). Spikes were sometimes seen at the vertices of PM2 particles. PR3 and PR4 phages are more similar to PM2 than ø6 in that they appear to possess a bilayered wall. Results reported in this thesis suggest this to be true for PR5 as well.

Lipid analysis of these lipid phages revealed a quantitative difference between phage and host lipids (table III and this thesis). These observations suggest that assembly of the viral capsid might involve a lipid biosynthetic mechanism specified by phage DNA. Hence, these viruses present themselves as potential tools in the study of lipid biochemistry. The PR5 group may prove to be especially useful because their host bacteria include a variety of E. coli , Pseudomonas and Salmonella strains whose biochemistry and physiology have been well studied and fully characterized.

MATERIALS AND METHODS

1. Bacterial strains and their maintenance

The bacterial strain used for routine propagation of the phage PR5 and as donor in the transfer of the R factor RP4 was a nalidixic acid resistant derivative of _E. coli_ K12 SA 1306 pro- met- (from Dr. K. Sanderson, University of Calgary) harboring the R factor RP4. The source of R factors used and their characteristics are listed in table IV.

P. aeruginosa strain PAO1 and its non-piliated derivative were originally obtained from Dr. D. E. Bradley, Memorial University, Newfoundland. _E. coli_ K12 JA53 pro- met- was from Dr. E. Meynell, University of Canterbury; and _P. aeruginosa_ 280 resistant to rifampicin was from Dr. Bryan. All other recipient strains were obtained from the culture collection of the Department of Medical Bacteriology.

Salmonella minnesota strains were kindly provided by Dr. K. E. Sanderson, Universty of Calgary. Their strain designations and chemotypes are listed in table XI.

All R^+ bacteria were routinely transferred every four weeks to agar containing a suitable antibiotic and the presence of R factors were confirmed using an antibiotic disc method (5). to detect appropiate antibiotic resistance.

TABLE IV

SOURCES OF R-FACTORS

R-factor	Source	Resistance
RP4	G. Jacoby, Massachusetts Gen. Hospital	Cb K T
RP1	M.H. Richmond, Bristol, England	Cb K T
RP9	" " " "	Cb K T
R1	" " " "	St Su Ch K Am
Sa	" " " "	St K G Am Ch Su
R57b	" " " "	Ch Su St Am
R112	Y. Chabbert, Paris, France	K
R113	" " " "	T
R931	L.E. Bryan, Edmonton	St T

Cb - carbenicillin, K - kanamycin, T - tetracycline,
St - streptomycin, G - gentamycin, Su - sulfonamides,
Ch - chloramphenicol, Am - ampicillin.

2. Chemical

All chemicals used were of reagent grade.

3. Bacteriophage

Phages PR3 and PR4 were kindly provided by Dr. V. Stanisich, Monash University. Phage PR5 was isolated from Edmonton sewage.

4. Media

Trypticase soy broth (TSB) or agar (TSA) (BBL).

Oxoid nutrient broth No. 2 (NB) or with 1.5% agar (NA).

Soft agar was 0.7% Noble agar (Difco) in NB supplemented with 1 mM calcium chloride.

Antibiotic plates for bacteria carrying RP4 were NA plates containing 100 ug carbenicillin and 50 ug kanamycin per ml.

Rifampicin resistant mutants were selected by plating inocula of rifampicin sensitive bacteria on NAP containing 100 ug per ml of rifampicin. Colonies appearing after 18 hours incubation were routinely resistant to higher than 100 ug/ml rifampicin.

Selection for R^+ recipients (transconjugants) was done on MacConkey agar or NA containing 100 ug rifampicin and 50 ug kanamycin per ml.

Mueller-Hinton agar (BBL) was used for drug resistance assay by the disc method.

5. Bacterial conjugation systems

A. Broth mating

Both donor and the rifampicin resistant recipient strains were grown to a density of 10^9 cells/ml (A 600=0.5, Spectronic 20, Bausch and Lomb) and were mixed at a ratio of 1 : 1 or 1 : 10, donor to recipient cells. Five ml of the mating mixture were added to a 150 ml medicine bottle and incubated flat side down at 37^0. The mating mixture was then diluted and plated on NAP or MacConkey plates containing rifampicin and kanamycin. Isolated bacterial colonies after overnight incubation were streaked once more on antibiotic plates to obtain pure clones of the transconjugant. These were grown in TSB and their drug resistance patterns were determined using the antibiotic disc method.

B. Filter mating for low frequency R factor transfer

Both the donor and the rifampicin resistant recipient strains were grown to a density of 10^9 cells/ml (growth temperature was 42^0 for _Salmonella_ species), and were mixed at a ratio of 1 : 1. Five ml of the mating mixture were filtered through a 25 mm diameter Sartorius membrane filter, pore size 0.45 u. This membrane was placed on NAP and incubated at 37^0 (42^0 for _Salmonella_ speies). After

overnight incubation, bacteria were eluted by suspending the membrane in a tube containing 5 ml of TSB and vigorously mixing the contents using a vortex mixer. Different dilutions of this bacterial suspension were plated on NAP or MacConkey agar plates containing rifampicin and kanamycin. Transconjugants were selected with the same procedure described above.

6. Bacteriophage isolation

The isolation of an RP4 specific phage from Edmonton sewage followed a method described by Olsen and Shipley (66). The centrifuged sewage supernatant was incubated with a log phase culture of E. coli K12 SA 1306 RP4 at 37^0. After overnight incubation the lysate was centrifuged at 5000 x g for 10 min to pellet bacteria. Different dilutions of the supernatant were plated on P. aeruginosa Pu21 RP4 using the double layered agar method (see 7) and spot tested (see 8) for lytic activities on R^+ and R- E. coli . Phage which formed plaques only on R^+ and not R- strains were eluted by stabbing the plaque with a needle; this single plaque elution was repeated twice. Phage isolated in this manner were propagated on E. coli SA 1306 RP4 as described in section 7.

7. Bacteriophage propagation and purification

The double layered agar technique (1) was used for preparation of high titre phage stock. Soft agar was

inoculated with a log phase culture of E. coli SA 1306 RP4 and bacteriophage was added at a multiplicity of infection (moi) of 0.001 phage per bacterium. The mixture was poured on NAP and incubated at 37^0. NB (10 ml) was added to the plates showing confluent lysis and was allowed to stand for 30 min.

The soft agar was scraped into a beaker and this was mixed with the broth on a magnetic stirrer for one hour to elute the bacteriophage from the agar. Agar and bacteria were removed from the mixture by centrifuging the preparation twice at 8000 x g for 20 min. The supernatant obtained was centrifuged in a Beckman L2-65B ultracentrifuge, rotor Ti 19 at 50,000 x g for 30 min at 4^0. The pellets were dispersed in a small volume of fresh TSB and centrifuged at 10,000 x g for 20 min at 4^0 to remove bacterial debris. The final supernatant was further purified in a CsCl step gradient.

A 5 step CsCl gradient was made with buoyant desities of 1.18, 1.22, 1.27, 1.31, and 1.35 g/ml. The phage was layered on the surface of the gradient. This was centrifuged in a Beckman L2-65B ultracentrifuge, rotor 39, at 35,000 rpm and 4^0 for 3 hours.

After centrifugation, a major phage band near the middle of the tube was clearly visible. The side of the tube was pierced with a hypodermic needle and the band collected into a syringe. Collected phage was stored in CsCl at 4^0 or dialysed against buffer solutions or distilled

water as required for individual experiments.

The R factor specificity of the phage preparation was confirmed by spot test (see 8). on R^+ and R- bacteria and its purity was established by electron microscopic observation.

8. Spot test for lytic activity

Double layered agar plates were prepared using 0.1 ml of a log phase culture of bacteria as inoculum. When the soft agar had set, a drop of a high titre phage stock (approximately 10^{10} PFU/ml) was put on the surface, and incubation was overnight at 37^0. Lytic activity was demonstrated by clearing of the bacterial lawn at the site of the phage drop.

9. Electron microscopic study of phage particles

Purified phage was dialysed against 2% ammonium acetate to remove CsCl. The phage was mixed with an equal volume of 1% phosphotungstic acid (pH 7). A drop of this mixture was added to a formvar coated grid and left for 2 min. Excess fluid was absorbed with filter paper and the grid was air dried and examined using a Philips 300 electron microscope. To determine the size of the phage particles, about 10^{11} per ml of polystyrene latex particles (Dow, 91 nm diameter) were mixed with the dialysed phage preparation, and the mixture stained with the abcve procedure. The size of the phage particles was then calculated by comparison with the size of

the latex particles on electron micrographs.

Bacteriophage adsorption was studied by mixing a log phase culture of bacteria with purified phage in TSB at a moi of 10 to 50. The mixture was incubated at 37° for 5 to 15 min. A drop of this mixture was placed on a piece of wax paper. A formvar-coated grid was suspended on the droplet for 5 min. The grid was then washed by suspending it on 3 droplets of 2% ammonium acetate. Subsequently, one drop of 0.5% PTA was placed on the washed grid for about 30 seconds. Excess fluid was removed with filter paper and the grid was allowed to dry in air for later electron microscopic observation.

10. Buoyant density of phage particles

A 0.5 ml volume of freshly prepared phage after differential centrifugation to remove most bacterial contaminants was mixed with 4 ml of CsCl solution in 0.05 M phosphate buffer, pH 7 (buoyant density 1.31 g/ml). This was centrifuged in a Beckman L2-65B ultracentrifuge, rotor SW 56, for 48 hours at 35,000 rpm and 20°. Five drop fractions were collected by peircing the bottoms of the centrifuge tube. Each fraction was assayed for infectivity and a standard volume was weighed. The fractions which corresponded to the peak of infectivities were collected, dialysed against 2% ammonium acetate, and negatively stained for electron microscopic observation.

11. Determination of phage nucleic acid content

A. RNA determination

A one ml volume of the purified phage in 1 x SSC (0.15 M NaCl + 0.015 M sodium citrate) was mixed with 3 ml of cold 0.5 N perchloric acid and centrifuged at 7000 x g for 10 min. The pellet obtained was resuspended in 1.5 ml 0.1% $FeCl_3.6H_22$ in concentrated HCl. A 0.15 ml volume of orcinol reagent (10% w/v in 95% ethanol) was added and the preparation was heated in boiling water for 30 min. The mixture was cooled in cold water and the absorbance was read at 660 nm (Beckman DB-G).

A standard curve relating absorbance at 660 nm and RNA concentration was constructed by using yeast RNA (Sigma type XI).

B. DNA determination

A 0.5 ml volume of purified phage in 1 x SSC was mixed with sufficient cold perchloric acid to obtain a final perchloric acid concentration of 0.5 N. After standing for 30 min in an ice bath, the mixture was centrifuged at 7000 x g for 15 min. After removal of the supernatants the pellet was resuspended in 3.5 ml 0.5 N perchloric acid and heated at 90^0 in a water bath for 15 min to extract DNA. After cooling, the suspension was centrifuged at 8000 x g for 10 min.

Three aliquots of the resulting supernatant were added to water to produce a final volume of 2 ml and then mixed with 0.5 ml 2 N perchloric acid. Three ml of diphenylamine reagent (1.5 ml concentrated sulphuric acid added to a 100 ml solution of 1.5% diphenylamine in glacial acetic acid) was added and the mixture was placed in a boiling water bath for 10 min. After cooling the absorbance was read at 700 nm and 595 nm (Beckman DB-G).

A standard curve was constructed using calf thymus DNA (Sigma, type I) by plotting the difference of absorbance at 700 nm and 595 nm against weight of DNA.

12. Determination of phage protein cotent

A 0.5 ml suspension of phage in 1 x SSC (approximately 10^{13} PFU/ml) was incubated with 1 ml of 1 N NaOH for one hour. This was diluted to 4 ml with water; and 3 different-sized aliquots were used to determine the protein content by the procedure of Lowry et al (50).

To 1 ml of sample was added 5 ml of freshly made copper reagent (prepared by mixing 50 ml of 2% sodium carbonate in 0.1 N NaOH with 1 ml of 1% sodium tartrate, then adding 1 ml of 0.5% $CuSO_4 \cdot 5H_2O$). The mixture was allowed to sit 10 min at room temperature, then 0.5 ml of 1 N Folin Ciocalteau reagent (Fisher) was added and the mixture was allowed to stand a further 30 min. Absorbance was determined at 660 nm (Spectronic 20, Bausch and Lomb) and a standard curve was constructed using bovine serum albumin (Sigma, fraction V).

13. Extraction of phage DNA

Phage DNA was extracted using the method described by Marusyk et al (53). Pronase (Sigma, type VI, specific activity 3-4 units/mg) preincubated 90 min at 37° was added to the purified phage suspension in 1 x SSC to a final concentration of 2 mg/ml . The mixture was incubated for 2 hours at 37° . Sodium dodecyl sulfate was added to a final concentration of 1% and the mixture incubated at 37° overnight.

DNA was extracted 5 times by adding equal volumes of distilled phenol saturated with 1 x SSC. The phenol was removed from the preparation by dialysis against 1 x SSC, and the absorbances at 260 and 280 nm were measured (Unicam SP 1800).

14. Extraction of bacterial DNA

Reference DNA was extracted from _P_. _aeruginosa_ 280 by the method of Marmur (52). Overnight culture from 15 NAP were scraped into 50 ml of 1 x SSC. This was centrifuged at 8000 x g for 10 min and the pellet resuspended in 25 ml 1 x SSC.

Two ml sodium dodecyl sulfate (25%) were added to the cells and incubated at 60° for 10 min. The lysed suspension was cooled, and perchloric acid was added to a final concentration of 1 M to dissociate protein from nucleic

acids. An equal volume of chloroform - isoamyl alcohol (24 : 1, v/v) was added and the mixture was shaken for 30 min at room temperature, then centrifuged to separate the layers. The upper aqueous layer containing the nucleic acid was carefully removed with a pipette and precipitated by adding 2 volumes of ethanol. The nucleic acid which spooled on the stirring rod was transferred into 10 ml of 1 x SSC. One ml of 0.2% ribonuclease (Calbiochem, grade A) was added to the DNA solution and this was incubated at 37^0 for 30 min. An equal volume of chloroform - isoamyl alcohol was added to remove protein which resisted earlier chloroform deproteinization. This was shaken for 15 min at room temperature and then centrifuged at 10,000 x g to separate the layers. The aqueous layer which contained the DNA was precipitated with 2 volumes of ethanol and transferred into a small amount of 1 x SSC as described above.

The absorbance of the DNA solution at 280 and 260 nm was measured to determine the protein and DNA ratio of the preparation.

15. Determination of buoyant density and base ratio of phage DNA

A. Equilibrium centrifugation method

Phage and reference DNA in 1 x SSC was suspended in CsCl (buoyant density 1.71 g/ml) to a final concentration of approximately 5 ug/ml. This was centrifuged using a Beckman

Model E Analytical Ultracentrifuge at 44,000 rpm for 25 hours at 25^0. Pictures were taken with an ultraviolet light source and traced with a densitometer.

Buoyant density of the phage DNA was calculated using the position of the reference DNA as a marker. The following equation was used:

$$P = Po + 4.2W^2(r^2 - ro^2) \times 10^{-10} g/ml^3$$

Po = buoyant density of standard DNA, which is 1.726 g/cm^3 for *P. aeruginosa* strain 280,

W = speed of rotation in radians per second which was calculated by the relationship W = 2 rpm/60,

ro = distance of standard DNA peak from centre of rotation,

r = distance of phage DNA from centre of rotation.

A linear relationship exists between buoyant density and GC content (77) which allowed the GC content to be calculated as follows:

$$P = 1.660 + 0.098(GC)$$

where P is the buoyant density and GC is the mole fraction of guanine + cytosine.

B. Thermal denaturation method

Nucleic acid was dialysed against 0.1 x SSC (sodium ion concentration was 21 mM; determined by flame photometry) and diluted to a concentration of approximately 12.5 ug/ml (A 260=0.25, Unicam SP 1800). In order to obtain spectral characteristics of DNA , this material was added to a 3 ml stoppered quartz cuvette having a 1 cm light path and placed in a Unicam SP 1800 spectrophotometer. Using an absorbance scale of 0 to 0.5 units, the sample was scanned at a wave length of 230 to 300 nm at a speed of 1 nm/sec and the absorbance plotted using a linear recorder operating at a chart speed of 10 sec/cm to see if the preparation adsorb at a single peak at 260 nm.

The wave length was then set at 260 nm and the temperature was raised at 1^0 per minute using a SP877 cell holder with an electric heating unit. The absorbance was recorded at a chart speed of 2 min/cm. Temperature readings indicated on the scale of a SP876 temperature program controller were recorded on the chart at every degree rise over the operating range of 50 to 93^0.

P. aeruginosa strain 280 chromosomal DNA was run as a control for melting point determinations immediately before phage DNA.

A linear relationship exists between the GC content and the melting temperature (71) :

$$\%GC = [\mathrm{Tan}(70.077 + 3.32Mg)][Tm - 175.95] + 260.34$$

where Tm is the temperature ^0C corresponding to 50% of the hyperchromicity and Mg is log $M[Na^+]$ of the buffer used (i.e. logarithm of molar sodium ion concentration in the sample).

The buoyant density was calculated from the %GC using Schildkraut's formula given in section A (77).

16. Molecular weight determination of phage DNA

A. Electron microscopic measurement

The monolayer technique described by Kleinschmidt (42) was used. DNA was extracted from a small amount of purified phage by the method described by Ralph et al (74) and also by an osmotic shock method (42).

Ten ul of phage was added to a solution containing 50 ul of EDTA(Disodium ethylenediaminetetraacetate)-phosphate buffer (0.01M EDTA in 0.01M PO_4, pH 7.8) and 100 ul 7.5N neutralized sodium perchlorate. After 5 min, the mixture was diluted with 1 ml of the EDTA - phosphate solution. A suitable volume of the DNA sample was added to 1 ml of a cytochrome C solution (Sigma type VI, 100 ug/ml) in 1 M ammonium acetate. A drop of this mixture was carefully delivered onto a glass ramp which led to the surface of a trough containing 0.25 M ammonium acetate (pH 7). Under

these conditions the cytochrome C spreads out to form a monolayer on the surface of the ammonium acetate. DNA is simultaneously spread over the latter surface because of the ionic interaction between cytochrome C amino group and nucleic acid phosphate group. The film was mounted onto a Formvar grid and stained with uranyl acetate (0.05 M in 50 mM HCl, freshly diluted 100 fold in 90% ethanol) for about 10 min. After washing the grid in isopentene, it was shadowed with platinum and palladium (80 : 20, w/w) for later electron microscopic observation.

In the osmotic shock procedure, 5 ul of the purified phage in 1 x SSC was suspended in a 1 ml solution of cytochrome C (Sigma type VI, 100ug/ml). Propanol was added to a final concentration of 0.005%. The mixture was gently floated down a ramp into a trough containing distilled water. The virus is osmoticallly shocked when it reaches the surface of water so that its DNA is extruded and adsorbed immediately to the protein film. This is mounted to formvar grids, stained and shadowed as described above.

Electron micrographs were taken and the negatives were projected onto a screen. The contour lengths of the DNA molecules were measured using a map measuring device. Magnification was calculated using a standard grating grid (2160 lines/mm, Ernest F. Fullam).

E. Sedimentaion velocity measurement

Sedimentation velocity measurement was made in a Spinco

model E Ultracentrifuge equipped with ultraviolet light source and optics.

A DNA solution of 1 ug/ml in 0.05 M Na_2HPO_4 (pH 7) was placed in a small well on the surface of the centerpiece, while the main centerpiece compartment was filled with 1 M NaCl. The specimen was centrifuged at 30174 rpm at 20^0. During acceleration, the DNA sample entered the main compartment to form a band above the supporting solvent. The movement of the DNA band was detected by ultraviolet absorption and pictures were taken at 8 min intervals. The run was stopped when the DNA band had sedimented half the tube length (approximately 40 min).

The observed sedimentation coefficient (Sob) in Svedberg units was calculated from the following relationship (88) :

$$Sob = (3.500 \times 10^{13}/rpm^2)(d \log rm/dt)$$

where rm was the distance of the peak of the band to the center of rotation and t was in minutes.

The observed sedimentation coefficient was corrected for solvent viscosity, temperature, and density using the correction factor $S20,W/Sob = 1.17$ calculated by Studier (88).

Since phage PR5 DNA was a small molecule as estimated by electron microscopic measurement , therefore, within

experimental error, the standard sedimentation coefficient can be assumed to be the same as the sedimentation coefficient at infinite dilution ($S^{0}20,W$). Allowing that assumption, the following relationship for native DNA at neutral pH can be applied to calculate the molecular weight of the DNA molecules (88).

$$S^{0}20,W = 0.0882\ M^{0.346}$$

17. Determination of strandedness of phage DNA by fluorescence assay

About 10 ul of the purified phage in 1 x SSC (A 260=1) was added to 2 ml of fluorescing solution (20 mM potassium phosphate buffer pH 11.8, 0.2 mM EDTA, 0.5 ug/ml ethidium bromide). Fluorescence was measure at room temperature using a Turner Spectrofluorometer 430 with excitation at 525 nm and emission at 600 nm. Flourescence of the blank solution without DNA and test preparations with DNA were determined at room temperature and after heating for 2 min at 98^{0} followed by cooling to room temperature. Strandedness of the DNA molecules was determined by comparing the fluorescence before and after the heat denaturation procedure (see section 5 in Results).

18. Solvent inactivation of phage infectivity

Purified PR5 phage in TSB (approximately 10^{6} PFU/ml) was mixed with equal volumes of chloroform or ether in

teflon capped tubes. The tubes were fixed to a platform shaking at about 2 oscillations per second. After 4 hours shaking at 37^0, they were centrifuged to obtain separate phases. The aqueous phase was assayed for surviving phages. A blank in which the solvent was replaced by TSB was processed similarly.

Phage morphology after solvent treatment was studied using freshly prepared phage in TSB at a concentration of 10^{11} PFU/ml. The phage were treated as described above and were negatively stained with phosphotungstic acid for electron microscopic examination.

19. Determination of phage lipids

The method described by Bligh and Dyer was used (7) for the extraction of phage lipids. All solvents used were redistilled and all glass ware was rinsed with methanol before use.

A. Lipid extraction and purification

Purified PM5 phage was dialysed against double distilled water and lyophilized in a New Brunswick Scientific B65 Freeze Dryer to a constant weight (Sartorious analytical balance). The dried phage material was suspended in 3.6 ml of water. To facilitate extraction of lipid from protein complexes, 0.4 ml of 20 mg/ml pre-incubated pronase (Sigma type VI, specific activity 3-4 units/mg) was added and further incubated for 3 hours at 37^0. Five ml of chloroform

and 10 ml of methanol were added to make a final ratio of chloroform : methanol : water of 1 : 2 : 0.8, v/v/v). The mixture was shaken at room temperature for 2 hours, then centrifuged at 5000 x g for 20 min. The pellet was resuspended in 4 ml water and re-extracted again using the same procedure.

To the combined suspernatant which contained 8 ml water, 10 ml chloroform and 20 ml methanol, a further 10 ml volume of chloroform and 10 ml volume of water were added to make a final ratio of 2 : 2 : 1.8, chloroform : methanol : water, (v/v/v). It was then centrifuged at 2000 x g for 10 min to separate the phases. The upper aqueous phase was decanted and the lower phase was carefully collected with a pasteur pipette. The remaining protein interface was washed once with methanol, centrifuged, and the supernatant combined with the lipid-containing lower phase.

The combined lipid extract was further purified by passing it through a 1 inch x 15 inch Unisil column (200 mesh, Clarkson Chemical Company) plugged at the bottom with glasswool. Lipid was eluted with 150 ml of methanol. The purified lipid extract was evaporated to dryness in a rotary evaporator (Buchler Instruments) at 50^0 under reduced pressure. The dried material was weighed using a Sartorius analytical balance and redissolved in 5 ml of chloroform and stored at -20^0 under nitrogen.

Lipids from E. coli K12 SA 1306 R- bacteria and from the same strain carrying the R factor RP4 were extracted

with the same procedures except that no pronase treatment was employed for the washed bacteria before extraction.

B. Thin layer chromatography of phospholipids

Chromatography plates 20 x 20 cm, precoated with Adsorbosil-5 to a thickness of 250 microns were obtained from Applied Science Laboratory, Inglewood, Calif. The plates were prewashed with chloroform-methanol-water, 65 : 25 : 4 (v/v/v) and reactivated in a 150^0 oven for one hour and cooled in a vacuum chamber. Using a micropipet, about 5 ul of the lipid extract in chloroform was applied 2 cm from the lower edge of the plate. The plate was placed vertically in a developing chamber with 180 ml of hexane-acetone (3 : 1, v/v). The solvent was allowed to run to the top of the plate. It was then dried in a vacuum chamber and placed in 180 ml of the second developing solvent, chloroform-methanol-water, 65 : 25 : 4 (v/v/v). The run was stopped when the solvent front was 1 cm below the solvent front of the first developing solvent. A phosphotidylethanolamine standard , kindly provided by Dr. P. G. Barton, Department of Biochemistry, was run with the phage and bacterial lipids.

Spots representing separated lipids on the chromatograms were visualized by putting the plates into a glass chamber saturated with iodine vapor or by spraying with 50% sulphuric acid followed by heating in a 180^0 oven.

For quantitative studies, the lipid extract was applied

to the chromatography plates as a series of small spots. Separate bands obtained after development were scraped off from the plates and eluted with 3 ml of methanol. A blank strip was also scraped from an area of the plate which contained no spots. The extraction was repeated two times. The combined extract was evaporated to dryness with a rotary evaporator at 56^0 under reduced pressure.

C. Determination of lipid phosphorus

Phosphorus determination was done using Martin and Doty's method modified by Kilroe-Smith (41).

The dried lipid materials eluted from individual spots on thin layer chromatograms were dissolved in 0.4 ml 60% perchloric acid. This was heated in a Bunsen burner flame until the solution turned colorless again. Water was added to a final volume of 3.6 ml, then 0.5 ml 10% ammonium molybdate solution and 5 ml benzene-butanol (1 : 1, v/v). The mixture was shaken for 15 seconds with a vortex mixer and centrifuged to obtain separate layers.

Three and one-half ml were transferred from the upper layer into a spectrophotometer tube and 1.5 ml of 3.2% H_2SO_4 in absolute ethanol and 0.5 ml stannous chloride reagent were added (10% $SnCl_2 \cdot 2H_2O$ in concentrated HCl freshly diluted 200 times with 0.5 M H_2SO_4). Color intensity was determined at 740 nm using a Spectronic 20 (Bausch and Lomb) Spectrophotometer.

A standard curve relating phosphate concentration to absorbance at 740 nm was prepared using anhydrous Na_2HPO_4 which had been dried overnight at 110^0.

20. One step growth experiments

A 0.9 ml volume of a log phase culture of host bacteria was infected with 0.1 ml of the phage at a moi of 10. The mixture was shaken gently in a water bath at 37^0. After 5 min, 0.1 ml of anti-PR5 serum was added and this was further incubated for 5 min. The mixture was centrifuged at 8000 x g for 10 min and the pellet was resuspended in 10 ml NB. This was diluted 100 fold with prewarmed NB and further incubated in the shaking water bath. Aliquots of 0.1 ml were taken at 1-2 min intervals to assay for infectivity.

21. R factor specificity of phage PR5

Three strains of bacteria, E. coli K12 SA 1306, E. coli K12 JA 53, and P. aeruginosa 280 harboring various R factor which covered seven incompatibility groups were used to test the R factor specificity of the phage. Bacterial host strains were confirmed to retain individual R factor (R^+) by using disc antibiotic sensitivity testing. Susceptibility to infection with PR5 phage was initially examined by a spot test for lytic activity. Bacteria that gave negative spot test results were tested for phage propagation in a broth culture of that test organism. A log phase culture of the R^+ strain (10^8 cells/ml) was infected with phage at a moi of

0.0001. The mixture was incubated at 37^0 in a shaking water bath for 6 hours, centrifuged, and the titre of the supernatant compared to the isogenic R- bacteria treated identically.

22. Bacterial host specificity

Different bacteria were infected with the R factor RP4 and spot tested with phage PR5 for lytic activity. Efficiency of plating (eop) was determined on bacteria that gave a positive spot test. Purified phage stock was diluted with NB to 10^2 - 10^3 PFU/ml and 1 ml was added to a tube of soft agar inoculated with 1 ml of a log phase bacterial culture of 10^8 cells per ml (1/10 dilution of a culture of A 600 = 0.5, Spectronic 20, Bausch and Lomb). The mixture was poured on NAP, incubated overnight at 37^0, and the plaque count determined. The eop was calculated by determining the ratio of plaque counts obtained with various bacterial hosts to that obtained with _P_. _aeruginosa_ Pu21 RP4, which gave the highest plaque count of all strains of bacteria tested.

Bacteria that were negative on spot test were assayed for phage propagation in broth. The precedure was the same as described in section 10B. An assay for phage adsorption was also done in which the incubation time was shortened to 20 min. The final titres were compared to the isogenic R- strains treated identically.

23. Infectivity of PR5 using cell wall mutants as hosts

Salmonella minnesota cell wall mutants were infected with RP4 by the filter mating technique described in section 5B. R^+ bacteria obtained from this mating were spot tested for lytic activity of the phage. All strains tested including the smooth strain failed to form plaques on spot testing. As a result, propagation and adsorption of the phage in broth was carried out using 10^8 bacteria per ml and an moi of 0.001 phage per bacterium.

The minimal inhibitory concentration of carbenicillin was determined with S. minnesota strains haboring RP4. The procedure was performed by inoculating 10^7 bacteria into 2.6 ml TSB containing serial dilutions of carbenicillin (20-80 mg/ml). After 18 hours of incubation at 37^0, the end point was determined visually.

24. Serological study of PR3, PR4 and PR5 phages

Anti-PR5 serum was prepared in rabbits by an initial subcutaneous injection of 10^{11} PFU/ml with Freund's adjuvant followed by intravenous injections of 10^{12} to 10^{13} phage weekly for 2 to 3 months until the antibody titre did not rise after repeated injections of phage. The titre was estimated from the K values of test sera (see below).

The rate of inactivation (K) of the phage by an antiserum was determined by adding 0.1 ml of 10^5 PFU/ml phage to 0.9 ml of a 1/10 or 1/50 dilution of antiserum in a shaking water bath at 37^0. Samples of 0.1 ml were taken out

at 2 min intervals and diluted 1/100 in broth to assay for surviving phage.

The rate of inactivation (K) was defined by the linear relationship (1):

$$K = 2.3D/t \log Po/Pt$$

where D is dilution of antiserum; t is min of incubation; Po is initial concentration of phage and Pt is concentration of surviving phage at time t.

At different time t1 and t2,

$$Kt1 = 2.3D \log Po/Pt1$$

$$Kt2 = 2.3D \log Po/Pt2$$

$$K(t1-t2) = 2.3D (\log Po/Pt1 - \log Po/Pt2)$$

$$= 2.3D \log Pt2/Pt1$$

$$\text{therefore, } K = 2.3D (\log Pt2 - \log Pt1)/(t1 - t2)$$

Therefore, if Pt is plotted against t, the rate of inactivation can be obtained by the formula

$$K = 2.3D \times \text{slope of the graph}$$

Anti-PR3 and anti-PR4 sera were kindly provided by Dr. D. E. Bradley, Memorial University. The K values of each antiserum against phages PR3, PR4 and PR5 were determined.

The K' values are of the ratio of the K value obtained with heterologous phages to that obtained with homologous phage.

RESULTS

1. Phage morphology

Electron microscopic examination of negatively stained preparations showed that phage particles had an icosahedral structure (Plate 1), with a diameter of 65-68 nm. Calibration of particle size was done by comparing Dow polystyrene latex particles of a known diameter (91 nm) with phage particles (plate 2). When empty of nucleic acid, a double-walled structure could often be seen (plate 3). Some particles appeared to have a tail (plate 4), but the frequency of these tailed phages varied considerably from one preparation to another. However, they did not appear to comprise more than 25% of the total phage in any preparation examined. Possible explanations of the last observation are given in the Discussion.

2. Receptor site

Electron micrographs of phage adsorbed to bacterial hosts showed that although adsorption occurred over the whole of the bacterial surface, the distribution was not uniform. Many more phage particles attached at the bacterial poles than elsewhere on the bacterial surface (plate 5). Phage were never observed to attach to pili. As strains of P. aeruginosa bear common pili, a specific sex pilus might be difficult to observe even with phage attached. As a result of this observation, strain PAO1 and the non-piliated

PLATE 1: Appearance of PR5 particles showing hexagonal outlines which strongly suggest an icosahedral structure. The particles shown here are tailess and appear to have full nucleic acid content. X 190,000.

PLATE 2: PR5 phages with latex particles. The latex particles have a known diameter of 91 nm. The diameter of PR5 was calculated to be 65-68 nm. Arrow indicates a latex particle. X 138,000.

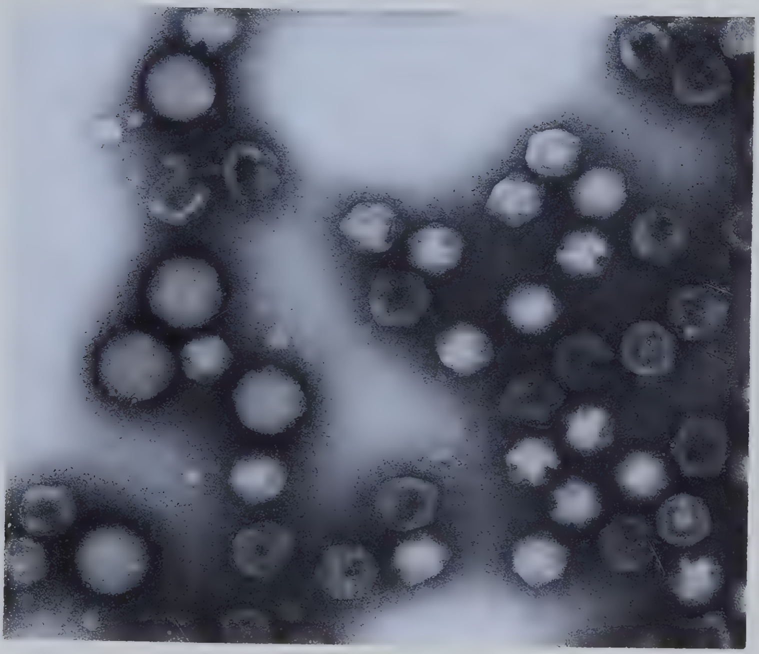


PLATE 3: A PR5 particle showing double-walled structure. The phage is apparently empty of its nucleic acid content. As a result, stain penetrates into the particle giving a darkly stained core. X 230,000.

PLATE 4: PR5 particles with and without tails. The tails appear to be of regular length approximately equal to the length of diameter of the phage particles. The 2 phages at the bottom right corner appear to be disintegrating. X 131,000.

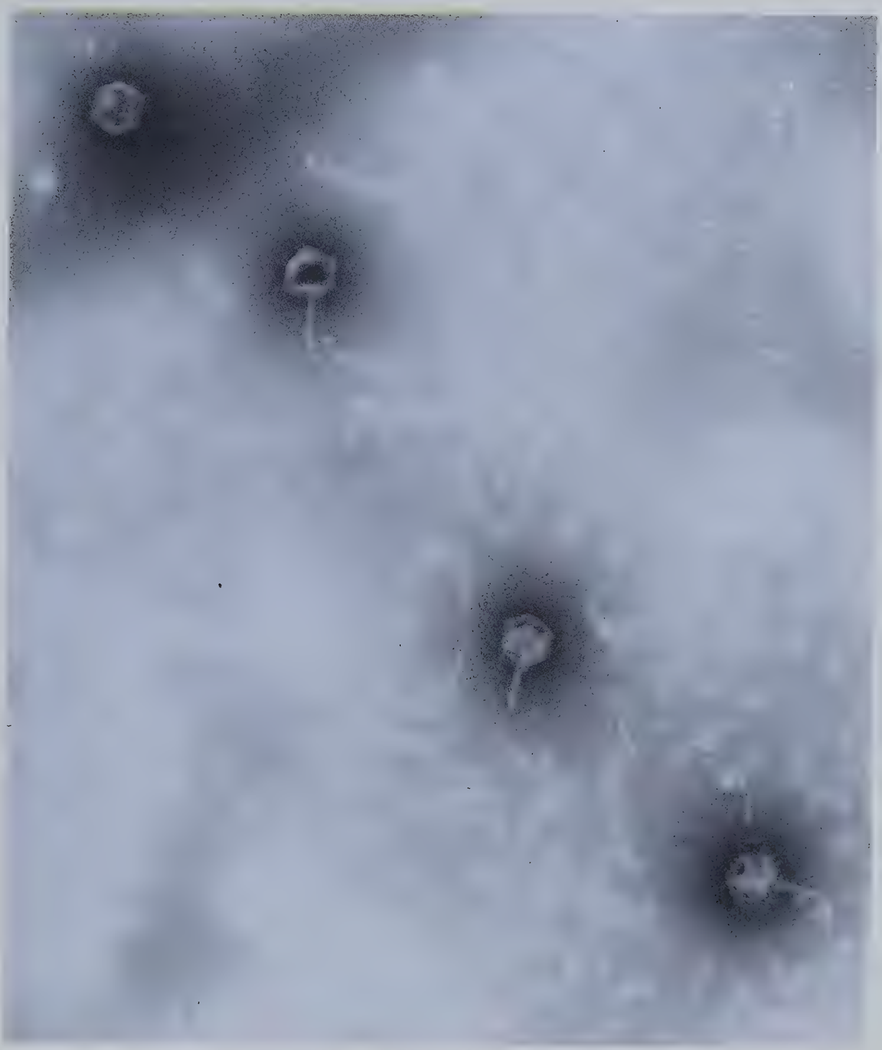
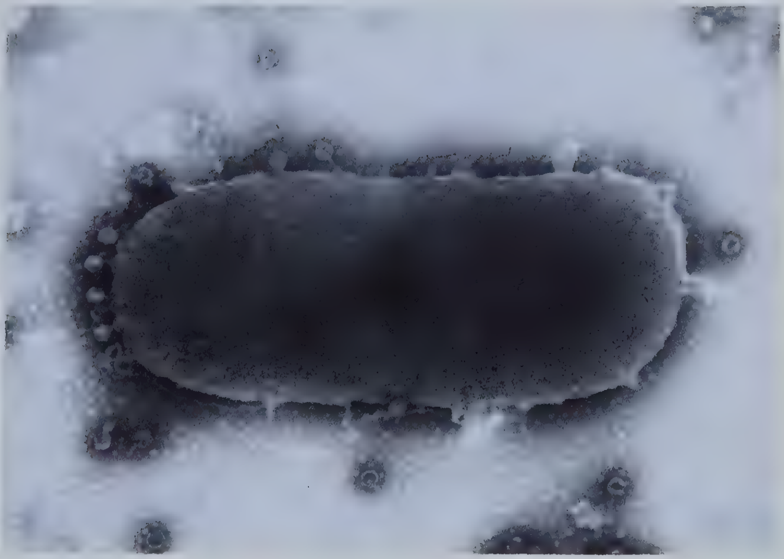


PLATE 5: PR5 adsorbed to a cell of the non-piliated strain of P. *aeruginosa* PAO1/PP7. The phages adsorb all over the surface of the bacteria but tend to aggregate at the pole. X 38,000.



mutant of that strain, PAO1/PP7 containing RP4 were examined for phage adsorption to pili. In both cases, such examination was repeatedly negative.

Phage were observed to be attached to bacteria in some instances by tails but in most instances apparently by the phage head. In the latter cases tails appeared to be absent from the attached phage (plates 6 and 7).

3. Buoyant density of phage particles

The result of a CsCl equilibrium centrifugation of a phage preparation is shown in figure 1.

Two peaks of infectivity were obtained which corresponded to buoyant densities of 1.26 and 1.31 g/ml respectively. Electron microscopic observation of aliquots of individual samples from these peaks showed that while both peaks contained phage with similar morphology, samples from the peak formed at higher buoyant density were heavily contaminated with bacterial debris. The samples from the other peak contained only phage when examined by electron microscope. Fractions from both peaks were spot tested onto lawns of R^+ and R- bacteria and were found to contain R specific phage.

In trial procedures the buoyant density of the apparently pure phage particle was therefore determined to be 1.26 g/ml. This information was used to construct CsCl step gradients so that the two bands were distinctly

PLATE 6: Phage PR5 adsorbed to a cell of the non-piliated strain of *P. aeruginosa* PAO1/PP7. Arrow shows a phage adsorbed by a tail. X 100,000.

PLATE 7: Phage PR5 of the same strain of bacteria. No tails can be observed with adsorbed phage particles. X 92,000.

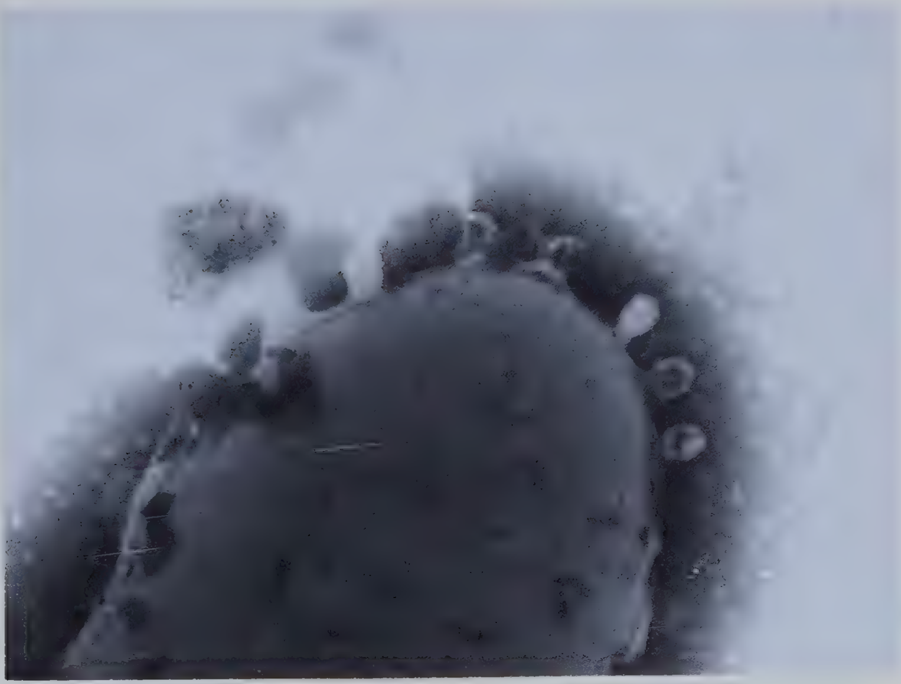
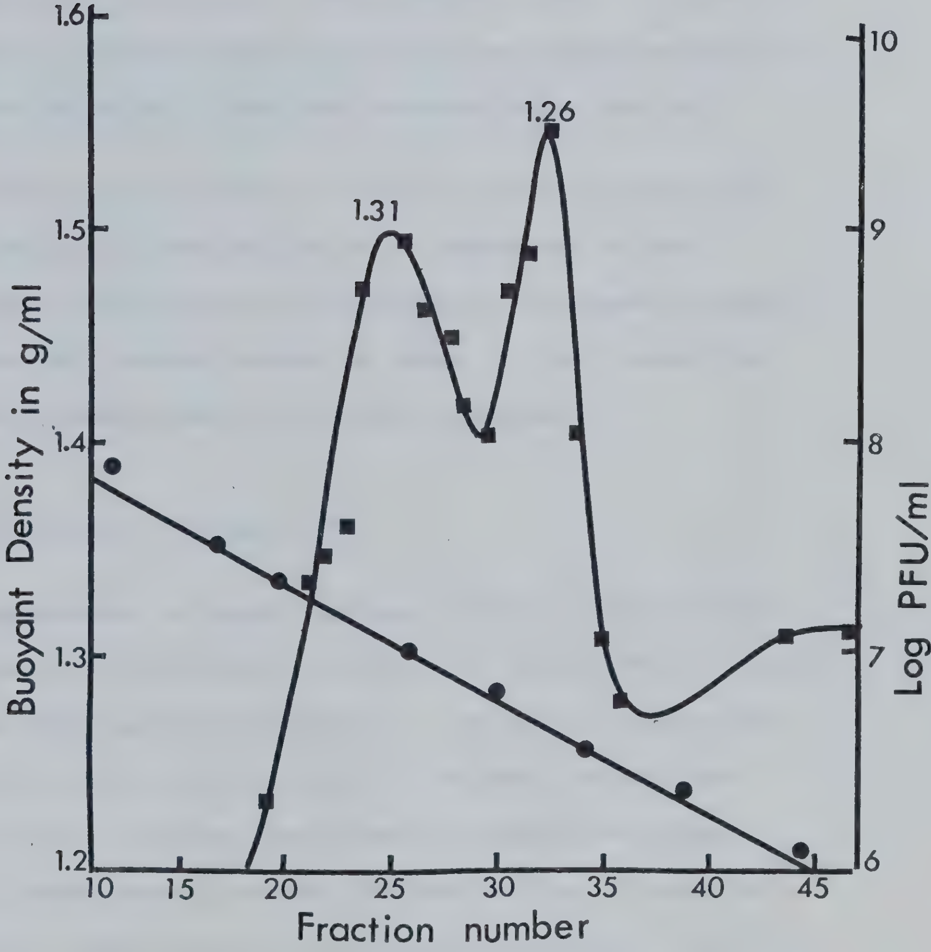


Figure 1: Equilibrium CsCl centrifugation of cell lysate partially purified by diffenential centrifugation. Fraction 1 (not included in the figure) corresponds to the bottom of the centrifuge tube.



separated by a band of intermediate buoyant density in later purification procedures.

4. Phage nucleic acid and protein contents

Phage preparation in 1 x SSC was examined by the diphenylamine and orcinol procedures to determine the nature of the nucleic acid present. The results given in table V showed that the diphenylamine reaction was positive and the orcinol test negative. Thus the nucleic acid was DNA.

The ratio of the weight of DNA to that of protein in phage particles was determined by a comparison of the content of DNA and protein of aliquots of the same sample of phage. From the results shown in table V, this ratio was found to be 19 parts DNA to 81 parts protein.

5. Characteristics of phage DNA

The absorption of phage DNA and reference DNA from P. aeruginosa strain 280 at 260 and 280 nm was determined using a Unicam SP 1800 Spectrophotometer. The ratio of the 280 : 260 absorption was less than 0.5. A single symmetrical absorption peak at 260 nm was formed by phage DNA when scanned for absorption between 230 and 300 nm (figure 3a). These results were consistant with highly purified DNA free of protein contaminations.

The U.V. absorption profile of CsCl density gradient equilibrium centrifugation of both phage DNA and P.

TABLE V

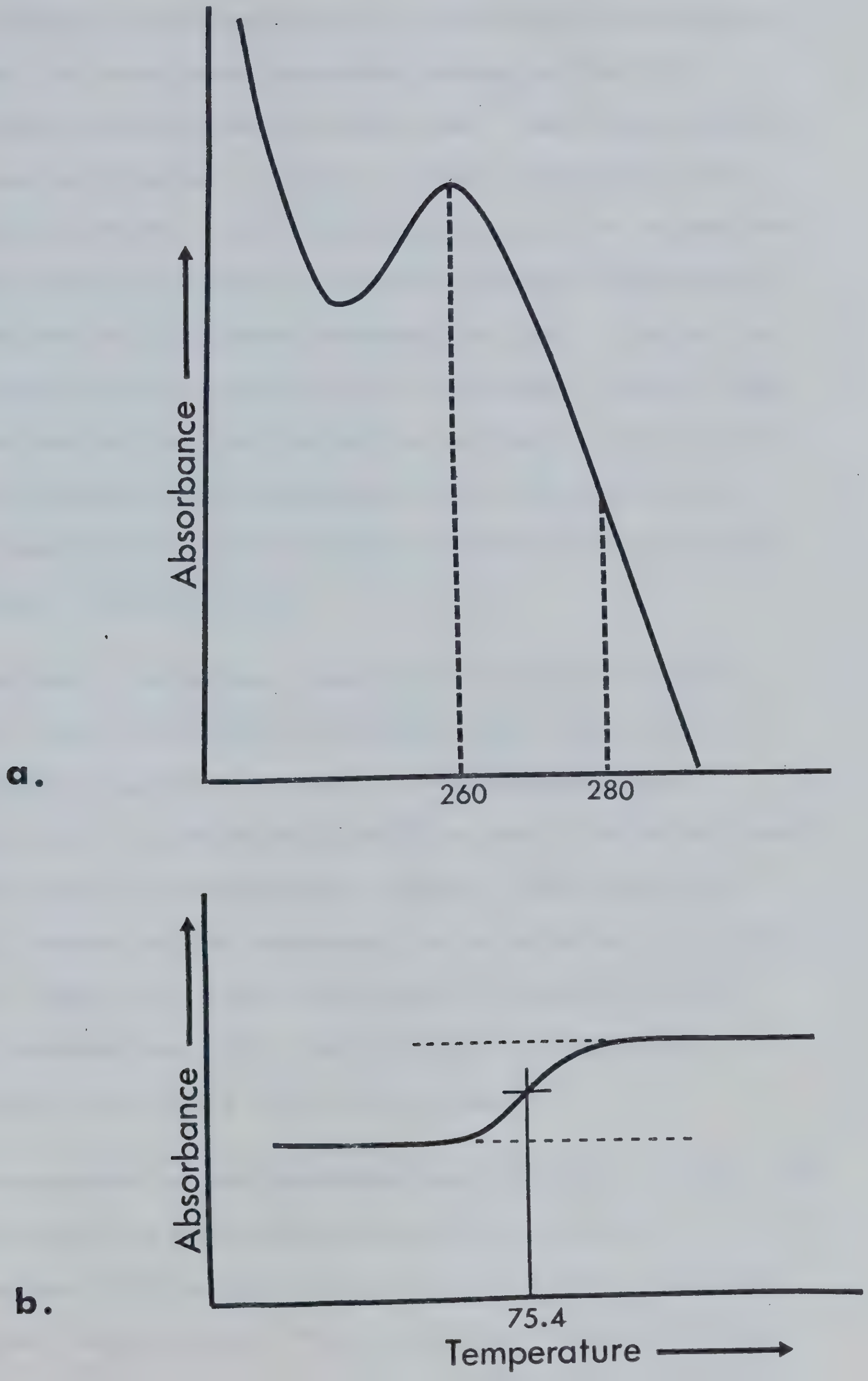
BIOCHEMICAL DETERMINATION OF NUCLEIC ACID AND PROTEIN CONTENT

	% by weight			Average
Protein[1]	81.2	80.8	81.4	81.1
DNA[2]	18.8	19.2	18.6	18.9
RNA[3]	0	0	0	

---

[1] Protein content was determined using the method of Lowry
[2] DNA content was determined by diphenylamine test
[3] RNA content was determined by orcinol test

Figure 3: a. Ultraviolet absorption profile of PR5 DNA at wavelengths 230-300 nm
b. melting curve at 260 nm.



aeruginosa strain 280 DNA is shown in figure 2. The buoyant density of the phage DNA was determined to be 1.708 g/ml.

On heating under controlled conditions (see methods), the phage DNA molecules denatured producing the U.V. absorption profile shown in figure 3b. The temperature at which the half-maximal increase in U.V. absorption (Tm) occurred was 75.4^0. The hyperchromicity of the heated DNA solution shown in figure 3 indicates the DNA was double-stranded and had undergone denaturation (86). Using the relationship given in section 15 in methods, the G+C base ratio of the DNA was determined to be 48.9%. The buoyant density obtained using these data was 1.708 g/ml which agreed exactly with that obtained by CsCl density gradient equilibrium centrifugation.

Electron microscopic examination of shadowed DNA revealed linear molecules of uniform size (plate 8). Photographic negatives of electron micrographs were projected at a standard magnification and 32 molecules were measured using a map measuring devise. The individual contour lengths of the molecules was determined to be 3.59 ± 0.3 um. Based on a value of 2.07×10^6 daltons/um for double stranded DNA (45), the molecular weight was calculated to be $7.4 \pm 0.6 \times 10^6$ daltons.

Measurement of the sedimentation velocity of phage DNA molecules gave an observed sedimentaion coefficient of 18.8 which became 22.02 after correction for solvent viscosity, temperature and density. The molecular weight calculated

Figure 2: Equilibrium CsCl centrifugation of DNA from phage PR5 and *P. aeruginosa* 280. The numbers are in g/ml.

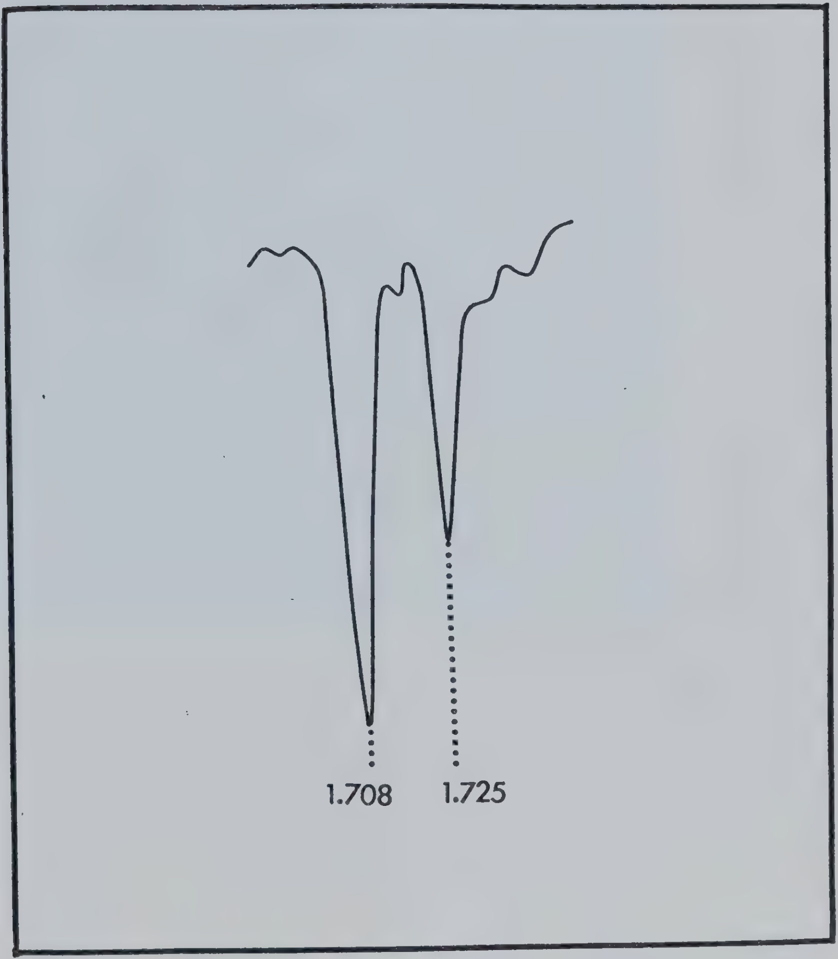
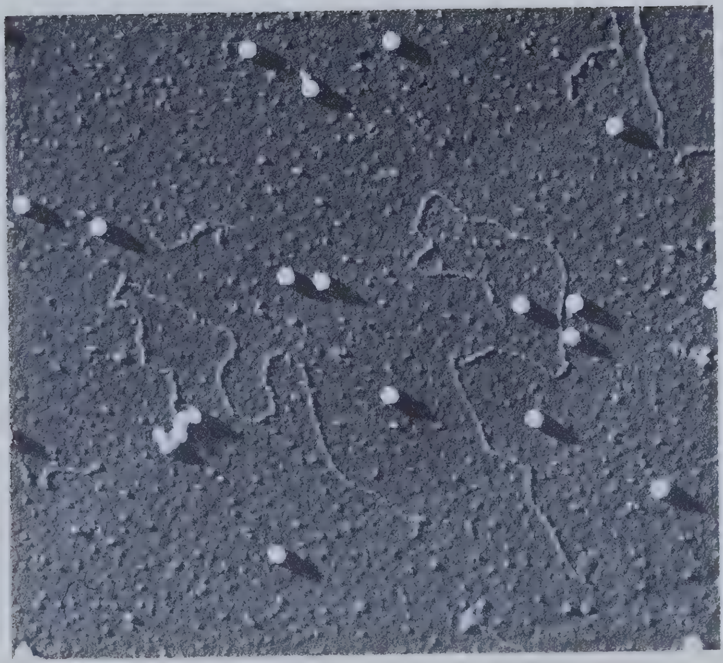


PLATE 8: Electron micrograph showing 2 linear molecules of phage PR5 DNA. The DNA has been released from the phage by osmotic shock and shadowed with palladium-platinum. X 43,000.



from Studier's relationship (88) was 8.4 x 10^6 daltons. This value should be considered approximate for it was calculated using the assumption that the corrected sedimentation coefficient S20,W was equal to the sedimentation coefficient at infinite dilution $S^0$20,W. In addition, the correction factor used to convert Sob to S20,W was an approximate value (18). However, since the assumption used to convert S20,W to $S^0$20,W seems valid (88), and because of the reasonable agreement with electron microscopically determinated estimation of molecular weight, and lack of facilities, no further determination was carried out.

Ethidium bromide fluorescence assay of phage DNA gave an initial fluorescence of 45 units. After heat denaturation at pH 11.8, the fluorescence was completely lost. This result indicates separation of the two strands of the DNA molecules since ethidium bromide only intercalates into duplex molecules and fluoresces. In the case of closed circular DNA molecules, heating in alkaline pH will not destroy the duplex structure of the molecules and hence the fluorescence is retained. The loss of fluorescence from the phage DNA therefore indicates absence of closed circular molecules. This result is in agreement with electron microscopic observation that the phage DNA molecules are linear.

6. Solvent inactivation of phage infectivity

Four hours of shaking a phage suspension with an equal

volume of ether at 37^0 produced 52% inactivation of phage infectivity compared to a phage suspension incubated with TSB. Incubation with chloroform produced 100% inactivation under the same conditions.

Electron microscopic examination of phage particles after either of the above solvent treatments revealed that the phage fell roughly into 4 morphological categories as shown in table VI and plates 9. Chloroform produced significantly more disruption of the entire phage wall than ether. Some disruption did occur in the TSB control, probably due to effect of shaking. The number of phage classified into the category of broken particles might have been underestimated because some phage particles which were badly broken may have been unidentified and therefore excluded from quantitation.

7. Phage lipids

The observation that phage PR5 was inactivated by organic solvents suggested the presence of lipid components. Therefore an attempt was made to quantitate and identify the lipids involved.

The phage was found to contain 17.5% by weight of lipid materials extractable by chloroform - methanol treatment. This was an estimation based on a single trial.

A thin layer chromatogram of lipids extracted from phage PR5 and uninfected R^+ and $R-$ host E. coli cells is

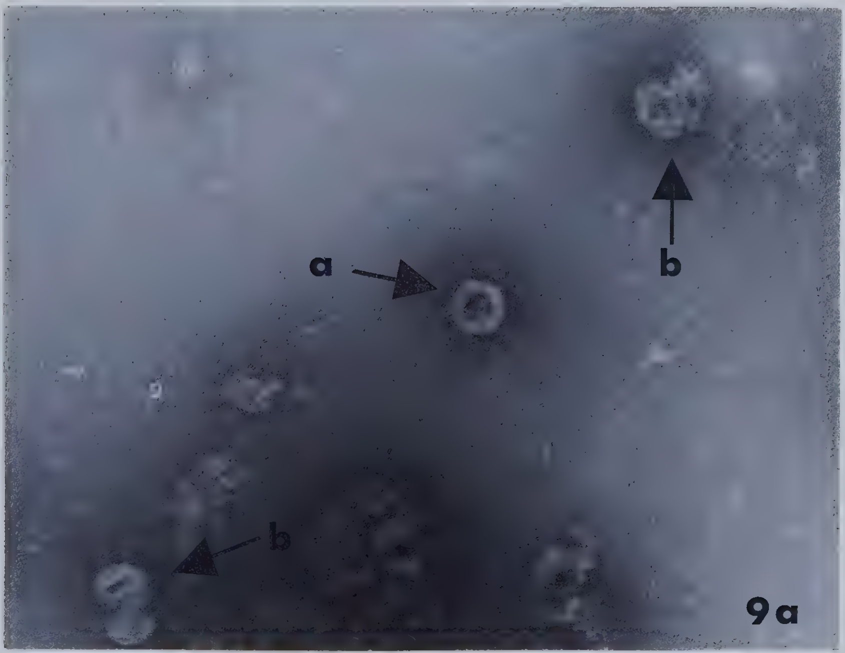
PLATE 9a: PR5 particles after chloroform treatment.

a shows a phage particle with empty head and intact wall and

b shows phages with entire wall broken.

X 138,000.

PLATE 9b: PR5 particles showing damaged inner wall after chloroform treatment. X 215,000.


a
b
b
9a

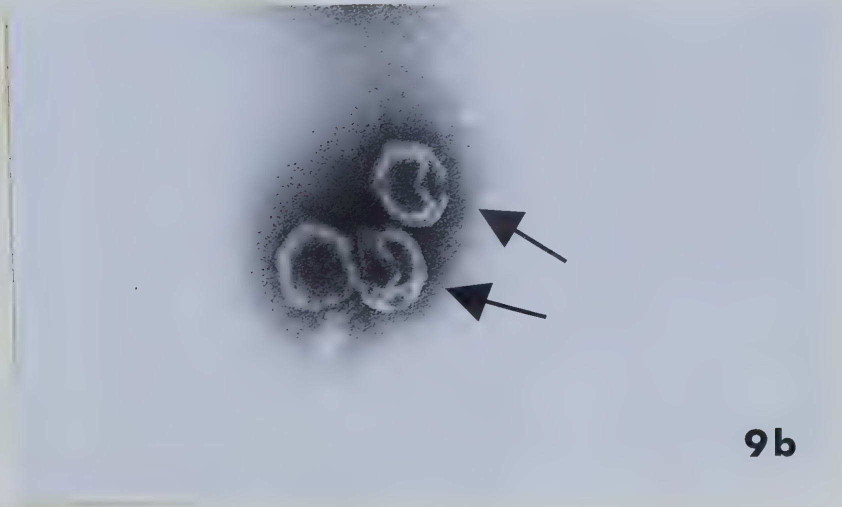

9b

TABLE VI
MORPHOLOGY OF PHAGE AFTER SOLVENT TREATMENT

	A	B	C	D	Total phage counted
TSB Control	50.6%	43.8%	2.8%	2.8%	114
Chloroform	2.9%	18.1%	22.3%	56.7%	238
Ether	39.7%	37.1%	9.5%	13.7%	199

A – Phage with full head and intact wall
B – Phage with empty head and intact wall
C – Phage with damaged inner wall
D – Broken phage particles

shown in plate 10. The phospholipid content of E. coli K12 and other strains has been widely studied and shown to contain 3 major components : phosphotidylethanolamine (PE), phosphotidylglycerol (PG) and cardiolipin (CL) (2,38,39). In this chromatogram, the identity of PE was confirmed using a PE standard. The position of PG and CL were determined by comparing the RF values of the respective unknown spots to the RF values of PG and CL from the lipid extracts of R- and R+ E. coli K12. The latter extracts were chromatographed on the same chromatogram as the phage extract. The chromatographic positions of PG and CL in bacterial extracts were determined from literature reports of chromatography of E. coli K12 extracts using the same chromatography system (2,72). The results showed that phage PR5 has the same phospholipids as host E. coli . With the R- host, 2 additional spots were detected near the solvent front. As these contained no detectable phosphorus, they were probably products from degradation of the phospholipids present.

Phosphorus analysis revealed that lipid content of the phage differed quantitatively from host E. coli. Table VII gives the results of the average of 2 determinations of phosphorus from individual chromatography spots. The phospholipids content of E. coli has been reported to be altered by growth conditions (19,82). This could account for the slight difference between the R+ and R- strains which had been grown under similar but not identical conditions. However, the content of each phospholipid in E. coli K12 cells is well within the range of values

PLATE 10: Thin-layer chromatogram showing separated phospholipids of _E. coli_ K12 SA 1306 R-, phage PR5, _E. coli_ K12 SA 1306 RP4 hosts, and a phosphatidylethanolamine standard (from left to right). First developing solvent was hexane-acetone 3:1 and second developing solvent was chloroform-methanol-water 65:25:4. The spots representing separated phospholipids on the chromatogram was visualized by spraying with 50% sulphuric acid followed by heating in a 180^{0} oven for 15 min.

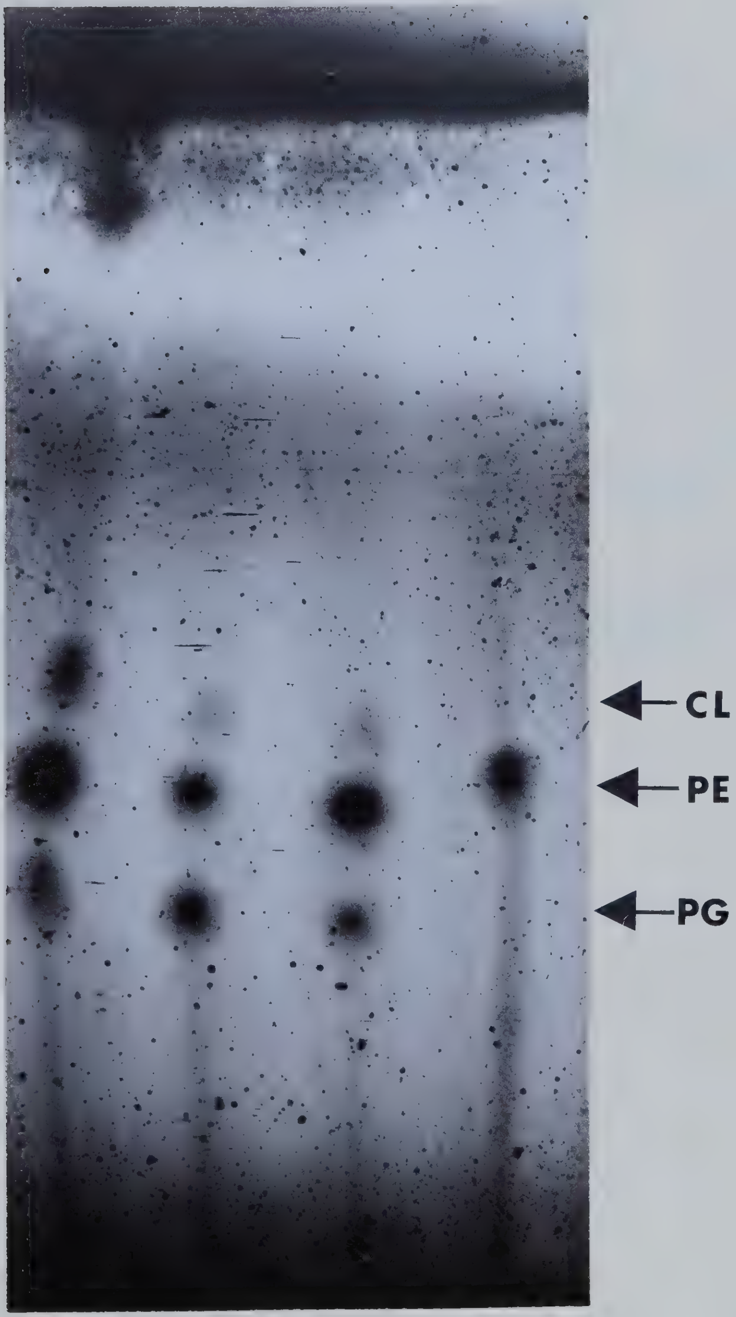

CL
PE
PG

TABLE VII

PHOSPHOLIPID CONTENT OF PR5 AND ITS HOST

Phospholipid[2]	% phosphorus by weight[1] PR5	E. coli K12 SA 1306 RP4	E. coli K12 SA 1306 R-
Cardiolipin	17	9.7	7.8
Phosphatidylethanolamine	41.3	73.7	78.9
Phosphatidylglycerol	41.7	16.6	13.3

[1] Individual phospholipids were separated by thin layer chromatography, eluted, and assayed for phosphorus content using a standard solution containing known amount of phosphorus.

[2] One mole of cardiolipin contains 2 moles of phosphorus while one mole of phosphatidylethanolamine and phosphatidylglycerol each contains 1 mole of phosphorus.

reported (2,38,39). The phage was shown to have significantly lower PE and higher PG and CL content than the host _E. coli_ . This suggests that the phage does not acquire its lipids ready-made from the host, but probably causes altered host bacterial phospholipid synthesis or membrane structure.

8. One step growth experiments

The one step growth curves of the phage in _E. coli_ SA 1306 RP4 and _P. aeruginosa_ Pu21 RP4 are shown in figures 4 and 5. The results show that with the same moi and under identical growth conditions, the phage had a latent period of about 48 min and rise period of about 30 min in both hosts. The average burst size with _E. coli_ host was calculated to be 106 which was considerably smaller than that obtained with the _P. aeruginosa_ host which was 280.

9. R factor specificity

The results of spot test for phage lytic activity (see methods) on bacterial hosts carrying various R factors are shown in table VIII.

The phage obviously lysed bacteria haboring the P1 group (RP1 and RP4) and the W group of R factors (Sa), and less clearly lysed the N group of R factors (R113). The results of broth incubation corresponded to the lytic activities shown in the spot tests. Using an initial titre of 1.5 x 10^4 PFU/ml and a 6 hour incubation period, the final titre showed a 10^4 fold increase with RP4, a 10^3 fold

Figure 4: One-step growth curve of PR5 in *E. coli* K12 SA 1306 RP4.

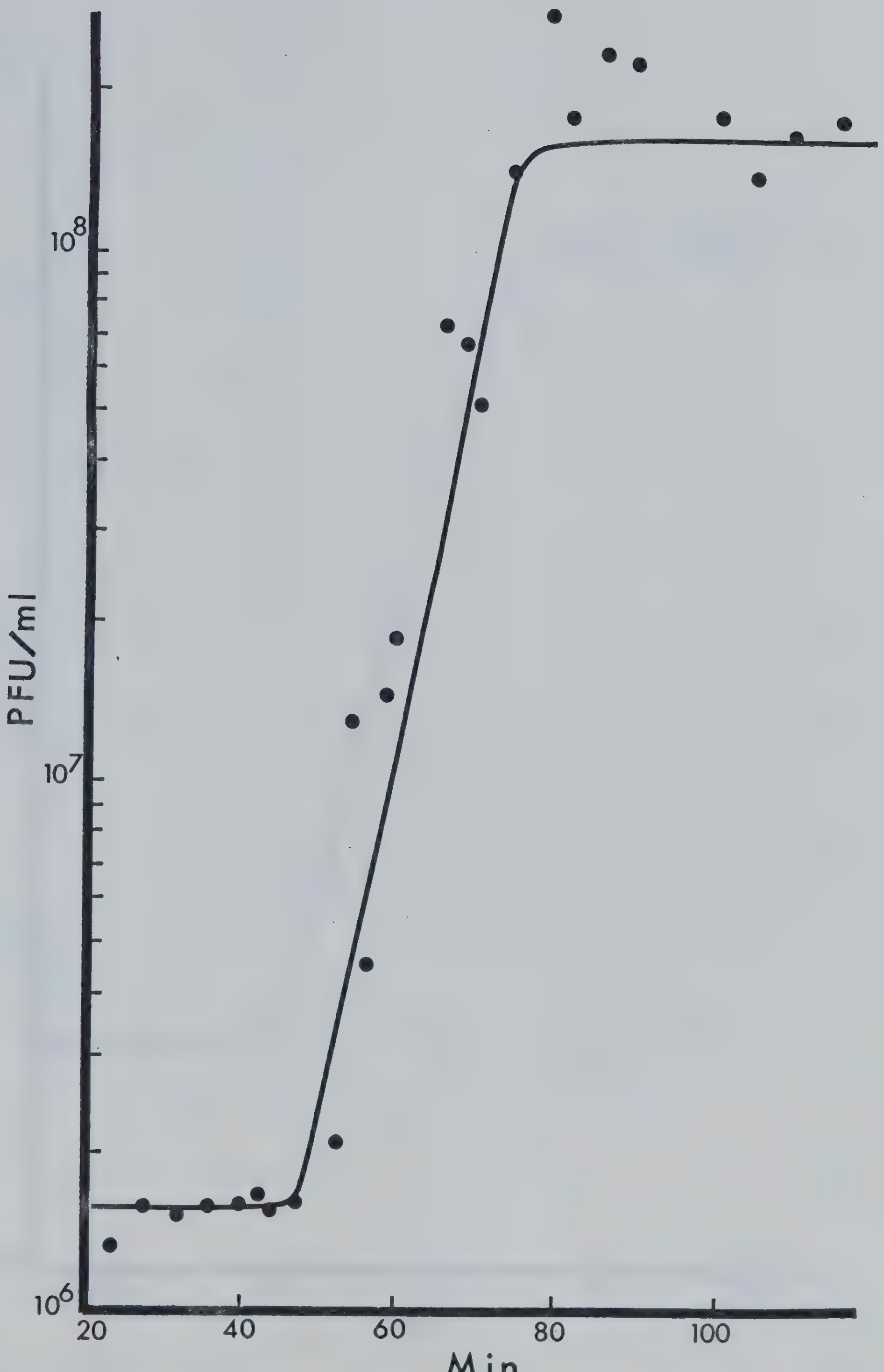


Figure 5: One-step growth curve of PR5 in P. *aeruginosa* Pu21 RP4.

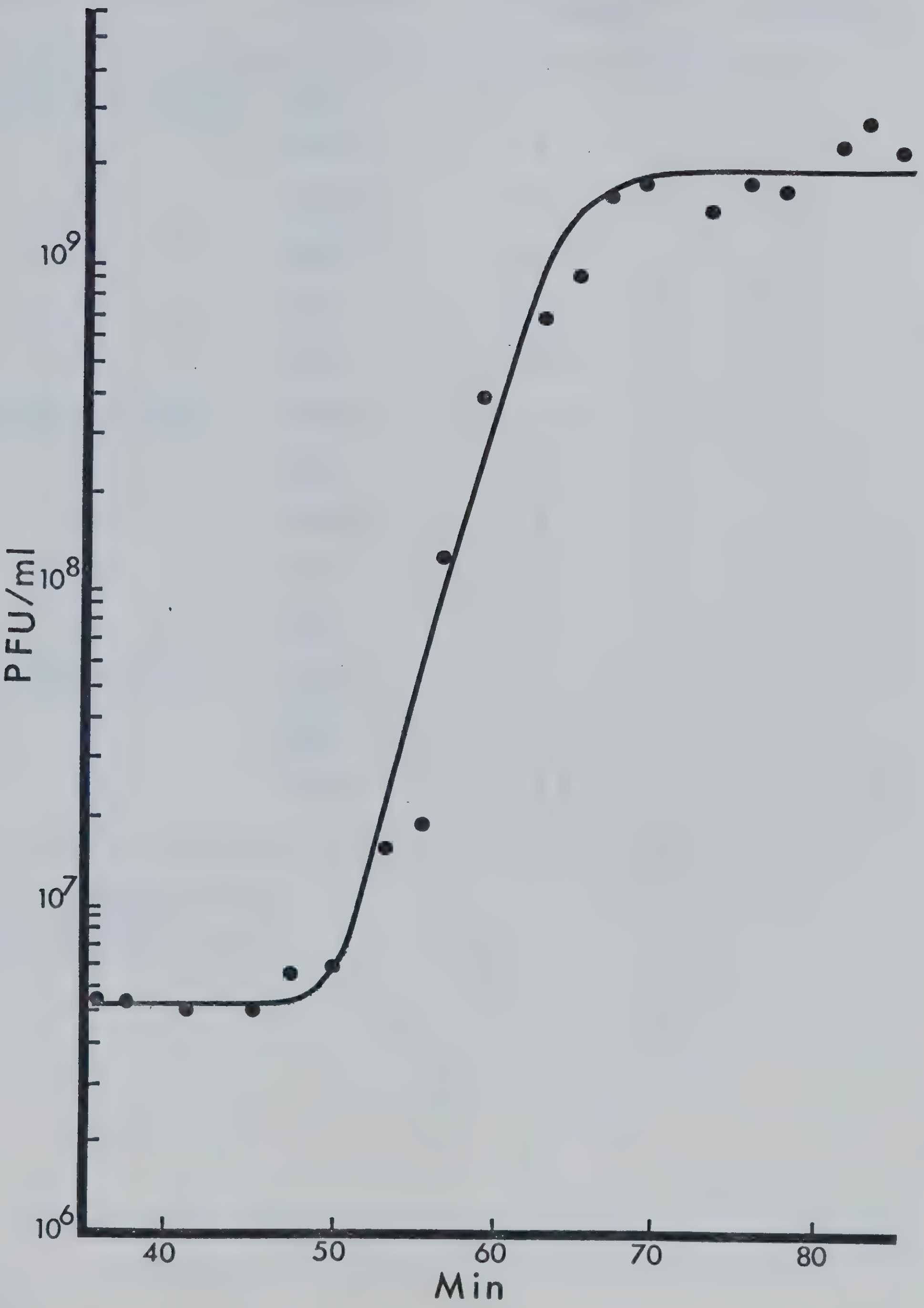


TABLE VIII

LYTIC ACTIVITY OF PR5 ON STRAINS CARRYING R-FACTORS

Bacterial strain	R-factor	Incompatibility Group	[1]Lytic Activity
E. coli K12 SA1306	none		–
"	R112	I	–
"	R113	N	(+)
"	RP1	P1	+
"	RP9	P1	–
"	RP4	P1	+
E. coli K12 JA53	none		–
"	RP4	P1	+
"	R57b	C	–
"	R1	F	–
"	Sa	W	+
P. aeruginosa 280	none		–
"	RP4	P1	+
"	R931	P2	–

[1] + = complete lysis
– = no lysis
(+)= partial lysis

increase with RP1 and Sa, and a 2 fold increase with R113. There was no significant difference in initial and final phage titres with the rest of the R factor tested when compared to titre obtained with the R- strain and the blank (containing no bacteria), indicating that neither propagation nor adsorption had occurred.

10. Bacterial host specificity

The R factor RP4 is known to be transferable among bacteria of several genera (20). R factor RP4 was transferred to several bacterial species to examine the effect on PR5 propagation. The results are summarized in table IX and X.

It can be seen that the phage PR5 was able to lyse a wide range of bacteria carrying the R factor with different efficiency and formed plaques of different morphology. Although plaque morphology is influenced by a number of factors, the plaques formed by an R factor dependent phage are expected to be influenced particularly by the degree of expression of the plasmid in different host bacteria. If the R factor is repressed in a portion of a bacterial population, these bacteria will lack the phage receptor site and become resistant to the phage, making the plaque turbid by their growth among susceptible bacteria.

Lysogeny is another possible cause for turbid plaque formation. However, no lysogenic R factor phage has been reported, therefore examination for lysogeny was not carried

TABLE IX

LYTIC ACTIVITY AND EFFICIENCY OF PLATING OF PR5 ON BACTERIA CARRYING THE R-FACTOR RP4

Bacteria	Lytic Activity	Eop	Plague Morphology
E. coli K12 SA1306	+	0.57	Tiny,clear
S. sonnei	+	0.05	Small,clear
S. typhimurium	+	0.65	Small,cloudy, irregular edge
S. thompson	+	0.03	Large, cloudy
Serratia	+	0.70	Small,clear
P. aeruginosa Pu21	+	1	Very large,clear
P. aeruginosa PA01	+	0.77	Very large,cloudy
P. aeruginosa 280	+	0.85	Small,clear
P. stutzeri	−		
K. pneumoniae	−		
P. mirabilis	−		
Aeromonas	−		

TABLE X

BROTH INCUBATION OF BACTERIA PRODUCING NO LYTIC ACTIVITY WITH PR5

Bacteria	% decrease in titre with R^+ host after 20 min incubation	Change in titre with R^+ host after overnight incubation
K. pneumoniae	1	No change
Aeromonas	32	66% decrease
P. stutzeri	88	16 fold increase
P. mirabilis	1	99.9% decrease
P. aeruginosa PAO1[1]	43	$2x10^4$ fold increase

[1] included as a positive control

out.

It can be seen that the phage infected P. aeruginosa strains most efficiently. With the 4 bacteria which exhibited no plaque formation, broth incubation was used to give additional information on their relationship with the phage. These results are shown in table X. Pseudomonas stutzeri showed a small increase of titre after overnight incubation suggesting that it could propagate the phage somewhat inefficiently. After 20 min incubation at 37^0, the phage and host bacteria in the preparation were centrifuged to pellet bacteria and absorbed phage. The supernatant was assayed for remaining phage activity and demonstrated an 88% drop in titre indicating that the majority of the phage had adsorbed to the bacterial cells. This shows that either the phage had a small burst size in this host or there was some restriction mechanism in the majority of the cells which prevented the phage from multiplying after adsorption.

Examination of Aeromonas and Proteus mirabilis in the same type of incubation experiment demonstrated a drop in titre of free phage both at 20 min and after overnight incubation. These results indicated phage adsorption but an apparent failure to propagate.

With Klebsiella pneumoniae , however, no change in phage titre was detected after 20 min and overnight incubation. It is probably that the thick slime coat of the bacteria prevented phage from reaching their receptor sites.

In all of the adsorption experiments, the change in phage titre was determined as the difference between that using R+ bacteria and R− isogenic bacteria. Therefore the possibility that this change in titre might have been a non-specific effect of the bacterial cell wall was excluded.

11. Infectivity with cell wall mutants

Since electron microscopic observation of the phage PR5 adsorbed to bacterial hosts demonstrated that receptor sites were located on the cell wall, S. minnesota mutants defective in various aspects of cell wall structures were employed to study the nature of the adsorption sites.

Results after overnight incubation of PR5 and several such mutants showed that the cell wall mutants did appear to exhibit a different degree of phage inactivation and thus phage adsorption (table XI). These results show that the smooth strain and the Ra mutants produced more than 99% inactivation while the rougher Rc mutant produced only 60%. No signigicant inactivation was observed when the strain was as rough as in mutant Rd. None of the mutants nor the parent propagated the phage. A strain of S. typhimurium (LT2, smooth) containing RP4 was infected with PR5 to act as a positive control and produced a 10^5 fold increase in the final phage titre. The failure of S. minnesota strains to propagate PR5 after adsorption is presumed to be for the same reasons discussed previously (see section 10).

R factor RP4 confers resistance to carbenicillin in

TABLE XI

CHARACTERISTICS OF S. MINNESOTA CELL WALL MUTANTS AND THEIR INFECTIVITY WITH PR5

Strain Designation[1]	Chemotype	Sugar Composition of Polysaccharide	Enzyme Defect in	% decrease of Phage[2]
SA 1639	Smooth	O-specific side Chain,KDO,Hep, Glc,Gal		99.8
SA 1640	Ra	KDO,Hep,Glc,Gal Glcn	O-specific Chain	99.6
SA 1642	Rc	KDO,Hep,Glc	Gal Epi	60
SA 1644	Rd1	KDO,Hep	Glc1 tran or UDP Glc synth	0

Abbreviations:-
KDO = 2-keto-3deoxyoctonic acid
Hep = heptoses
Glc = glucose
Gal = galactose
Glcn = glucosamine
Gal Epi = UDP-galactose epimerase
Glc1 tran = glucosyl(1) transferase
UDP-Glc synth = uridine diphosphate galactose synthetase

[1] All bacteria tested including mutant and smooth strain did not form plague on spot testing with PR5

[2] Decrease in phage titre was determined by comparing the final titre of PR5 incubated overnight with strain carrying RP4 to the final titre of the phage incubated with the isogenic strain

these strains of _S. minnesota_. The effect of the change in cell wall polysaccharide associated with the various rough mutants on this carbenicillin resistance was examined by determining the MIC of carbenicillin for each of the R^+ mutants (table XII). The roughest mutant Rd did show lower carbenicillin resistance than any other mutant. Possibly severe deficiencies of the outer membrane of _S. minnesota_ enables the drug to reach its target site in the peptidoglycan layer more readily. However, no reasonable explanation has been found to explain the observation that the smooth strain with complete polysaccharide structure had the same MIC as Rc strain.

12. Serological relationship of phages PR3, PR4 and PR5

In spite of the use of a commonly employed immunization program, a low titre antiserum to the phage was obtained in rabbits. The most likely explanation of this result is that the phage is a poor antigen. The relatively high lipid content of the phage is in keeping with this conclusion. In addition, other individuals using the similar phages have also obtained low titre antisera.

Figure 6 shows the rate of inactivation of anti-PR5 serum against PR3, PR4 and PR5 phage. It is clear that it inactivated the three phages at different rates, the highest K value was obtained with the homologous phage PR5. The results obtained using anti-PR3 serum also showed different K values with the three phages with the highest K again

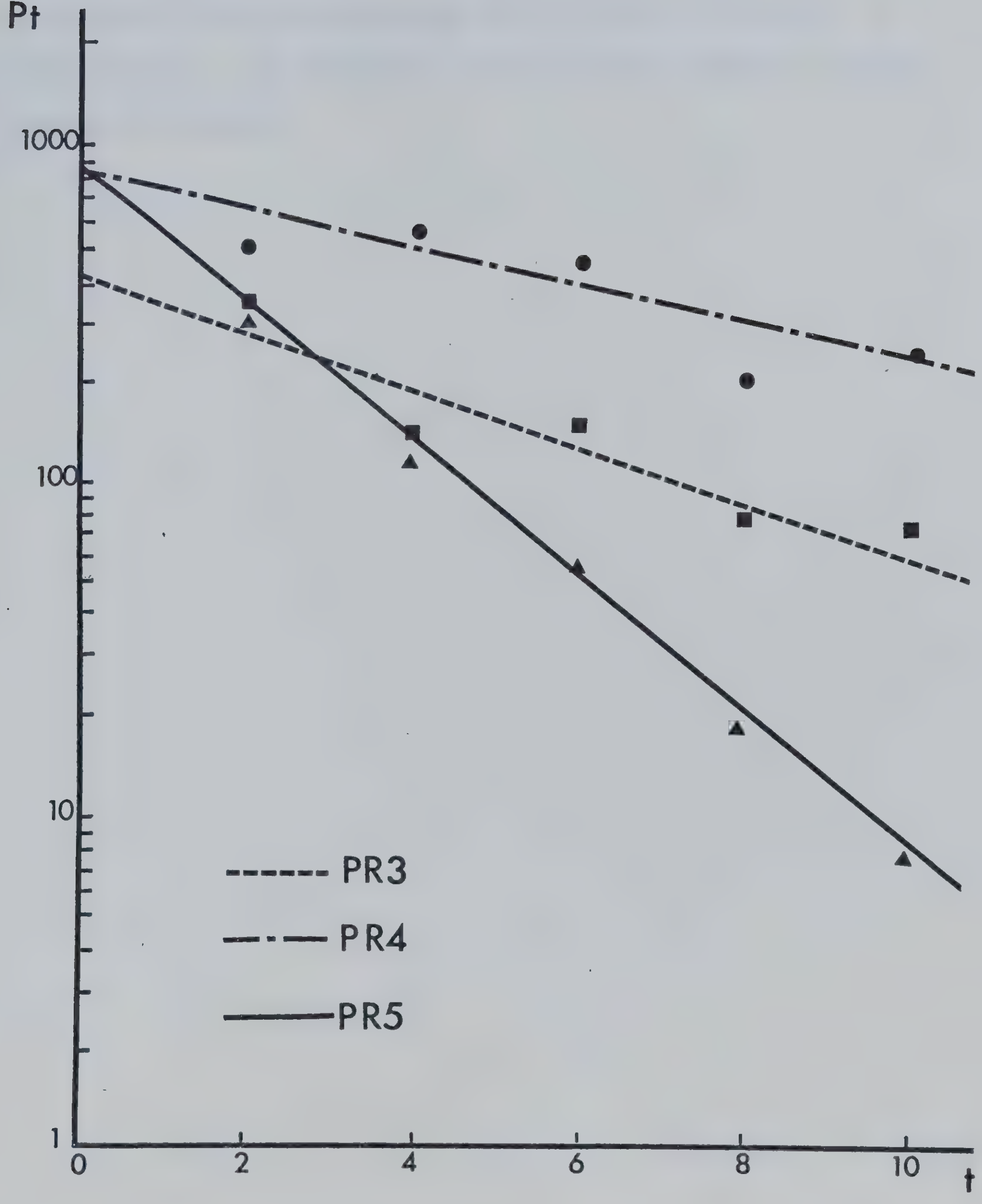
TABLE XII

MIC OF CARBENICILLIN FOR SALMONELLA MINNESOTA CELL WALL MUTANTS

Strain	Chemotype	MIC (mg/ml Carbenicillin)
1839 RP4	Smooth	60
1640 RP4	Ra	80
1642 RP4	Rc	60
1644 RP4	Rd1	40

S. typhimrium LT2 R- strain was included as a control and was found to have an MIC of 4-8 ug/ml

Figure 6: Rate of inactivation of anti-PR5 serum against PR3, PR4 and PR5 phages.



obtained with the homologous phage. With anti-PR4 serum, no clear cut relationship could be obtained due to its low K value and lack of specificity.

Table XIII shows the K' values of anti-PR3 and anti-PR5 sera calculated from the average of 3 or more K values. It is concluded that the three are serologically related though not identical phages.

TABLE XIII

RELATIVE RATE OF INACTIVATION CONSTANTS (K') FOR ANTI-PR3 AND ANTI-PR5 SERUMS WITH PHAGES PR3, PR4 AND PR5

Serum	K for homologous phage	K' values		
		PR3	PR4	PR5
Anti-PR3	7	1	0.44	0.76
Anti-PR5	45	0.47	0.28	1

DISCUSSION

The present study has revealed several characteristics of the phage PR5 which mark it as belonging to a new class of bacterial virus. Its biochemical composition, plasmid specificity and the manner of adsorption not only distinguish it from all other male phage studied, but also distinguish it from another very large group of morphologically similar isometric phage with non-contractile tails (10,91). The tail of PR5, however, presents some uncertainty because of its absence from a large number of phage particles including apparently intact phage heads with full nucleic acid content. The same is true of PR3 and PR4 phages. It has been reported that phage particles with full head and intact tail are extremely hard to find with these 2 phages (12). The possibility that either the tailed or non-tailed phage is a lysogenic contaminant of the preparation is unlikely because the 2 species have identical head morphology and buoyant density. They also have similar nucleic acid since DNA extract of phage preparations containing both species gave one single sharp peak in CsCl density gradient equilibrium centrifugation and have one thermal denaturation temperature indicating similar G+C base ratios. Two other possibilities exist. One possibility is that the tailess particles are incomplete phage produces by defective intracellular multiplication and assembly. Defective phage particles have been reported to be present in large quantities in Bacillus mycoides phage No. 1 (90) and the S. typhimurium phage P1 (4). In both cases, the

abnormality lies in the size of the phage head. Although a similar defect of phage tails has not been reported, it is reasonable to allow for this possibility. Another possible explanation is that the phage head and tail have been detached during preparation and staining procedures. Similar observations were obtained when crude lysates were studied by electron microscopy, which suggests that the detachment was not due to the preparation procedures. But the possibility that the damage might occur during the staining procedures have not been excluded. However, no firm conclusion can be drawn.

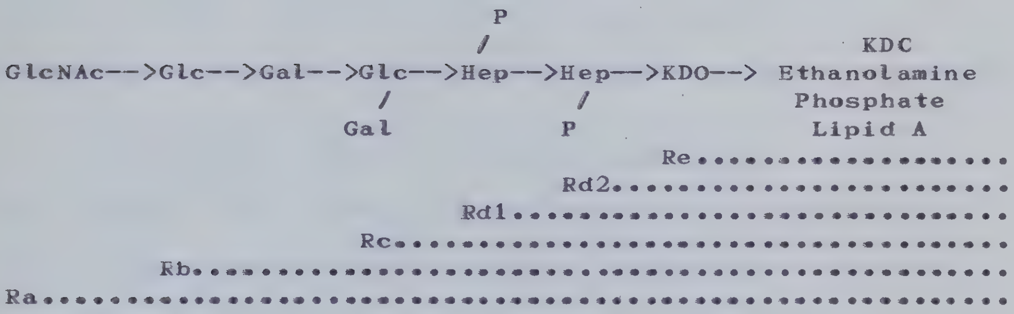
The problem is further complicated by the electron microscopic observations that the majority of the adsorbed phage particles appeared to be tailess. This raises the question whether the tail is an intrinsic component of the phage structure, as it appears that these tails are not functionally indispensable. Studies on phage T1 and T5, isometric phages with long non-contractile tails, have revealed that they adsorb to their host cells with the tips of their tails (3). Experiments using purified receptors extracted from _E_. _coli_ cell wall demonstrated that these receptor combined irreversibly with the tips of the T5 tail, causing release of phage DNA (96). Therefore in these cases, it is clear that the non-contractile tail is functionally involved in phage adsorption and nucleic acid transfer during the initial stage of phage infection. Performance of this function by an additional structure on the phage capsid has not been reported. However,

speculations based on the present stage of knowledge on PR5 appear to be futile. An answer to this question has to await further studies.

As has been pointed out previously, several other phages have also been isolated which infect bacteria carrying RP4. Except for PRR1, which adsorbs to R pili, the remaining phages Pf3, PR3 and PR4 all have somatic receptor sites like PR5. This indicates that bacteria infected with RP4 receive at least 2 new antigens which are absent in R- bacteria, a pilus antigen which adsorbs PRR1 and a cell wall antigen which adsorbs somatic R phages. Indeed, a new heat stable O-somatic antigen has been discovered in _E. coli_ which have received RP1, an R factor very similar to RP4 (Pitt, personal communication in (12) The exact chemical nature of this antigen has not been identified. It is likely that this O-antigen is the receptor for RP4 somatic phages.

Some additional information about the nature of this receptor has been obtained from the results of the experiment using _S. minnesota_ cell wall mutants. A comparison of the degree of phage adsorption (see results) and lack of completeness of their lipopolysaccharide structure (figure 7) shows that strains lacking O-specific polysaccharide (Ra) adsorbed the phage as efficiently as the smooth strain with complete cell wall structures. Adsorption still occurred to a level at 60% of that of the smooth strain with an Rc mutant which lacks the outer core

Figure 7: Proposed simplified structures of different R polysaccharides in *S. minnesota* (51).



GlcNAc = N-acetylglucosamine
Glc = glucose
Gal = galactose
Hep = heptose
KDO = 2-keto-3-deoxyoctonic acid

polysaccharides but retains glucose, heptose and keto-deoxy-octonic acid (KDO). No adsorption took place with the Rd1 mutant which has only heptose and KDO. This suggests that the hexose sugars are involved in the structure of the receptor site. Glucose, in particular, might be a structural component of the new antigen synthesized by RP4.

The receptor sites of other somatic phage are fairly well known. By selective removal of various components of the _E. coli_ cell wall, it has been demonstrated that the receptors for T2 and T6 are situated in the outer lipoprotein layer, while the receptors for T4, T3 and T7 are in the underlying lipopolysaccharide layer (97,98). With the use of multi-resistant mutants, the common receptors for T3, T4 and T7 has been identified as the sugar L-gala-D-mannoheptose. The receptors for the _Salmonella anatum_ phage e15 has also been identified as a polymer of trisaccharides mannose-rhamnose-galactose. Specificity of binding is due to the α linkage of the galactose to the mannose of the next trisaccharide (49). The receptors for T5 have been extracted from _E. coli_ using very weak alkali (98). Purification of this active material yield spherical particles composed of two layers. The core polysaccharide also bound T3, T4 and T7 after the outer lipoprotein layer had been removed by phenol. However, treatment with phenol destroyed the activity against T5, which indicated that the receptors for T5 are situated in the lipoprotein layer.

It is apparent that the receptors for PR5, like those

for T4, T3 and T7, involve the lipopolysaccharide structure of the host cell wall. Unlike the T-receptors which are components of host cell wall, PR5 receptors are plasmid dependent and therefore might be biochemically different from pre-existing cell wall components. However, it is possible that the same kind of methods can be used to study the PR5 receptors, hence the nature of the RP4-directed somatic antigen.

It has been shown that the plasmid specificity of PR5 is not restricted to the P group of R factors. The phage can also infect bacteria carrying the W and N group. Hybridization experiments of R factor DNA has demonstrated a 50% homology between the P group and the N group, and also a slight homology between the P group and W group of R factors (36). It is possible that the PR5 receptors are determined by the homologous portion of the plasmid DNA. PR5 however, does not lyse RP9, which is another member of the P group. The reason is not known. Although hybridization experiments showed a high degree of homology between RP9 and RP1, the 2 plasmids are not identical (36). It is possible that the receptors are not expressed in bacteria carrying RP9, or the plasmid simply lacks the genes that code for the PR5 receptors.

Of the 2 lipid-containing phages PM2 and ø6, PR5 is more similar to PM2 in the absence of an external envelope and in the amount of lipid content (see results and table III). All 3 phages have similar phospholipids to those of

their host bacteria except PM2, in which traces of phosphatidic acid has been found which were absent from its host. The percentage of each individual phospholipid, however, was very much different from their host bacteria. All 3 showed a marked increase in phosphatidylglycerol and a decrease in phosphatidylethanolamine content.

Virus infection has been known to produce changes in the lipid metabolism in animal cells. Lipid synthesis may be stimulated or suppressed, or, in some cases, only selective classes of lipids are effected (43). The difference in the amount of individual lipids in the phage from that of their host bacteria suggests that similar effects might have been produced by infection with phages.

Treatment of ø6 (10^{10}PFU/ml) with 25% ether for 7 to 8 seconds produced a 10^6 fold decrease in phage infectivity (93). Although different condition has been employed to study the effect of solvent on PR5 (see methods), it is apparent that PR5 is considerably more resistant to ether than ø6 (see results). Since both phages have similar lipid contents, the absence of a lipid envelope in PR5 and the difference in ether sensitivity suggest different locations of the lipid constituents. This possibility is supported by studies on PM2 which also lacks a lipid envelope.

X-ray structure analysis and electron microscopic studies of the PM2 particles (32) indicated that the phage lipid is arranged as a bilayered structure with a low electron density non-polar inner region. The polar groups

are separated from the external protein coat by another low electron density region which, when negatively stained, gives rise to a darkly stained band beneath the stain excluding protein coat. Ocassionally, when the particle is empty of nucleic acid, stain enters the central core defining a second stain excluding layer which is the lipid bilayer. Therefore when negatively stained, the empty phage particles have a double-walled appearance.

As has been mentioned earlier, empty PR5 particles give a similar double-walled appearance when negatively stained. This suggests that PR5 might also have a bilayered structure similar to that of PM2. Its low sensitivity to ether might therefore be explained by the relative inaccessibility of the solvent to the lipid layer due to an external protein coat.

Lipids in animal viruses are thought to be involved in infection of host cells (43). The role of lipids in these bacteriophage is unknown. It is uncertain whether infectivity remain with ø6 particles after their lipid envelopes have been removed by solvent treatment (23).

Two phages PR3 and PR4 have been isolated in Australia which have similar plasmid specificity as PR5. In addition, they also have double stranded DNA, similar buoyant density and morphology in electron micrographs. PR3 however, differs from PR4 in plaque morphology (12). Neutralization studies using anti-PR5 and PR3 sera was able to demonstrate considerable antigenic differences between the 3 phages,

although cross reactions exist (see results).

An analogous situation occurs among the RNA phage. These phages are similar in plasmid specifity, morphology, and in other biochemical properties (see introduction). Serological studies, however, provided evidence that they are different phages. M12 and MS2, for instance, gave similar K' value (0.88) with heterologous anti-MS2 and anti-M12 sera; however, using anti-f2 serum, different K' values were obtained for M12 and MS2, showing that they are different phages (80). Analysis on their protein coats showed that the two phages have identical amino acid composition (46). The same study also demonstrated that f2 and R17 capsid protein differs from these by one amino acid replacement. This finding correlates with serological observations that f2 and R17 reacted with anti-MS2 serum giving 0.54 and 0.72 K' values and with anti-M12 serum giving 0.41 and 0.71 K' values respectively (80). Therefore these serological tests are able to detect even slight difference in phage protein structure. The same kind of serological studies have been used to study various mutants isolated from the wild type phage λ (29) and showed that certain mutants were neutralized at only 0.25 of the rate for the wild type phage λ. Therefore it is possible that the group of RNA phage might all be serological mutants of the same phage.

Since there is no well defined criteria of similarity and dissimilarity between 2 phages using K' values, in the

case of the PR phages, one can only conclude from information available that PR3, PR4 and PR5 are related but different phages. Further information can be obtained from biochemical analysis on composition of phage protein and DNA hybridization experiments.

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